ARGUMENTS/REMARKS

Claims 1, 2, 4-82, and 84-88 constitute the pending claims in the present applications. Claims 1, 2, 4-59, 63-66, 68-82, and 84-88 are currently under consideration. Applicants respectfully request reconsideration in view of the claim amendments and the following remarks. These amendments do not narrow the scope of the claims. Issues raised by the Examiner will be addressed below in the order they appear in the Office Action.

Claim rejections under 35 U.S.C. 112, second paragraph

Claims 1, 2, 4-59, 63-66, 68-82 and 84-88 are rejected under 35 U.S.C. 112, second paragraph, for allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which applicants regard as the invention. Applicants have amended the claims to obviate the rejections, with the exception of two rejections, which will be addressed below.

(1). Claim 84 and 85 are rejected for allegedly being directed to subject matter that is "not the goal of the instant application". Applicants respectfully traverse this rejection to the extent it is maintained in light of the amended claims.

The Office Action stated that the present invention is "directed to such detection utilizing host cells with a readout system which is not capable of being auto-activated". Applicants submit that this statement does not accurately describe the claimed invention, which recites methods that screen out those host cells carrying genetic element capable of auto-activation. The claimed methods detect interacting molecules by utilizing host cells with a readout system that is capable of being auto-activated. Such a readout system may allow counter-selection (claim 1), or may allow visual differentiation (claim 2), to differentiate cells that express auto-activating molecules from those that do not express auto-activating molecules. Claims 84 and 85 are directed to kits that utilize the latter readout system, which allows one to visually differentiate host cells with auto-activating molecules from those without. Accordingly, Applicants submit that claims 84 and 85 are clear and do not depart from the goal of the instant application. Reconsideration and withdrawal of the rejection is respectfully requested.

(2) The claims currently under consideration are generally rejected for allegedly being vague and indefinite for using abbreviations for gene names. Applicants traverse this rejection to the extent it is maintained in light of the amended claims.

The definiteness of claim language must be analyzed, not in a vacuum, but in light of (1) the content of the particular application disclosure, (2) the teachings of the prior art, and (3) the claim interpretation that would be given by one possessing the ordinary level of skill in the pertinent art at the time the invention was made. *See, e.g., In re Marosi,* 710 F.2d 799, 218 U.S.P.Q. 289 (Fed. Cir. 1983); *Rosemount, Inc. v. Beckman Instruments, Inc.,* 727 F. 2d 1540, 221 U.S.P.Q. 1, (Fed. Cir. 1984); *W.L.Gore & Assocs., Inc. v. Garlock, Inc.*, 721 F.2d 1540, 220 U.S.P.Q. 303 (Fed. Cir. 1983); and *Atmel Corp. v. Information Storage Devices, Inc.,* 198 F.3d 1374, 53 U.S.P.Q. 2d 1225 (Fed. Cir. 1999).

In the present case, Applicants submit that, at the time of the filing of the present application, the gene names recited in the claims were routinely used as such in the pertinent art. To expedite prosecution, Applicants have amended the relevant claims to include the full names wherever possible. For the remaining genes, URA3, LYS2, ADE2, TRP1, LEU2, HIS3, sacB, CAN1, CYH2, rpsL, lacY, lacZ, tetA, sacB and luxAB, Applicants submit that these are not abbreviations, but the terms by which these genes were referred to in the art. Their usages were abundant in textbooks as well as in scientific journal articles at the time of filing of the present application. For example, Chapter 13 in Short Protocols in Molecular Biology, edited by Ausubel et al. (John Wiley & Sons, Inc., 4th Ed., 1999), refers to URA3, LYS2, TRP1, HIS3, LEU2, CYH2, CAN1 and lacZ. See Table 13.4.2 on page 13-18, and page 13-28, a copy of which is enclosed with this Reply as Exhibit 1. For another example, C.K. Murphy et al refers to lacY, rpsL and sacB in a 1995 paper, *Gene* 155: 1-7 (1995), a copy of which is enclosed with this Reply as Exhibit 2. Indeed, the specification of the present application cited this paper (See page 16, second paragraph of the Specification). Likewise, TetA and luxAB were described in two articles published in 1998 and 1993, respectively. See Chen et al., *J. Biol. Chem.* 273: 653-659 (1998); and Kondo et al., *Proc. Natl. Acad. Sci. USA.* 90: 5672-5676 (1993), a copy of each of which is enclosed with this Reply as Exhibit 3 and 4, respectively. Furthermore, numerous issued U.S. patents refer to these gene names in the same way. For example, U.S. Patent No. 5,283,173, the Fields patent that the Examiner cited in the pending Office Action, contains numerous reference to

these genes, including, for example, URA3 and LEU2. See the bottom of column 10. Thus, one with ordinary skill in the art would have understood the meaning of these gene names. Accordingly, reconsideration and withdrawal of the rejections is respectfully requested.

Claim rejections under 35 U.S.C. 102 and 103

Claims 65 and 66 are rejected under 35 U.S.C. 102 for being allegedly anticipated by Fields et al. (U.S. Patent No. 5,283,173, "Fields"). Applicants traverse this rejection to the extent it is maintained in light of the amended claims.

The standard for anticipation is that "a claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference." *Verdegaal Bros. v. Union Oil Co. of California*, 814 F.2d 628, 631, 2 USPQ2d 1051, 1053 (Fed. Cir. 1987). Claim 65 and 66 are directed to kits containing at least one set of host cells, at least one genetic element, and visual means for visually differentiating host cells in different activation states of readout system, wherein the visual means include digital image capture, digital storage, digital processing and/or digital analysis. Fields does not teach the use of digital analysis and thus does not anticipate claim 65 and 66. Accordingly, reconsideration and withdrawal of the rejections is respectfully requested.

Claims 65 and 66 are further rejected under 35 U.S.C. 103 for allegedly being unpatentable over Fields in view of Augenlicht (U.S. Patent No. 4981783, "Augenlicht"). Applicants traverse this rejection to the extent it is maintained in light of the amended claims.

To establish a *prima facie* case of obviousness, there must be "some suggestion or motivation, either in the reference themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings." MPEP 2143. Applicants submit that the motivation to combine Fields and Augenlicht was absent at the time of the filing of the present application. Fields, which discloses methods of detecting interacting molecules, does not describe the use of digital visual means for image capture, storage, processing and analysis, an element of claims 65 and 66. Augenlicht, on the other hand, describes digitalization of gene expression data useful for diagnosis and prognosis of diseases. Contrary to

what the Office Action suggested, Augenlicht does not provide motivation to combine digitalization of gene expression data with methods of detecting interacting molecules. The language that the Examiner pointed to for supplying motivation, i.e., column 3, line 56, through column 4, line 2, merely discusses the advantages of analyzing large numbers of sequences, rather than a single or a small number of genes, for distinguishing phenotypes. It does not suggest combining digitalization with methods of detecting interacting molecules. According to MPEP, "the mere fact that references can be combined or modified does not render the resultant combination obvious unless the prior art also suggests the desirability of the combination". MPEP 2143.01. Applicants submit that without the motivation to combine Fields and Augenlicht, a *prima facie* case of obviousness cannot be established. Accordingly, reconsideration and withdrawal of the rejections is respectfully requested.

CONCLUSION

For the foregoing reasons, Applicants respectfully request reconsideration and withdrawal of the pending rejections. Applicants believe that the claims are now in condition for allowance and early notification to this effect is earnestly solicited. Any questions arising from this submission may be directed to the undersigned at (617) 951-7000.

If there are any other fees due in connection with the filing of this submission, please charge the fees to our **Deposit Account No. 18-1945.** If a fee is required for an extension of time under 37 C.F.R. § 1.136 not accounted for above, such an extension is requested and the fee should also be charged to our Deposit account.

Respectfully Submitted,

Date: December 15, 2003

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Table 13.4.2 Cloned Yeast Genes

Yeast gene	Length of sequenced fragment (bp)	Map position in yeast genome	Selectable phenotype in *E. coli*	*E. coli* strain[a]	Selectable phenotype of wild type in yeast	Common nonreverting mutant alleles	Selectable phenotype of mutant alleles in yeast[b]	GenBank file name
ARG4	2296	8R	ArgH$^+$	JA209	Arg$^+$	None	None	YSCARG4
ARS1	1453[c]	4R	None	n.a.	None	n.a.	None	YSCTRP1
CAN1[d]	n.s.	5L	None	n.a.	Can^s	*can1*-100 *can1*-11	Canavanine sulfate^r	YSCCAN1
CEN3	627	3	None	n.a.	None	n.a.	None	YSCCEN3
CEN4	2095	4	None	n.a.	None	n.a.	None	YSCCEN4
CYH2[e]	1393	7L	None	n.a.	Viability[f]		Cycloheximide^r	YSCRPL29
GAL1-GAL10 regulatory region	907	2R	None	n.a.	None	n.a.	n.a.	YSCGAL
HIS3	1822	15R	HisB$^+$	BA1	His$^+$	*his3*-Δ1 *his3*-200	None	SCHIS3Y
LEU2	2230	3L	LeuB$^+$	JA300	Leu$^+$	*leu2*-3,112	None	YSCLEU2
LYS2	n.s.	2R	None	n.a.	Lys$^+$	*lys2*-Δ1	α-aminoadipate^r	n.a.
TCM1[g]	1529	15R	None	n.a.	Viability[f]	n.a.	Tricodermin^r	YSCRP13
TRP1	1453[c]	4R	TrpC$^+$	JA300	Trp$^+$	*trp1*-289 *trp1*-Δ901	None	YSCTRP1
URA3	1170	5L	PyrF$^+$	DB6656	Ura$^+$	*ura3*-52 *ura3*-Δ1	5-fluoroorotic acid^r	YSCODCD

n.s. = not sequenced; n.a. = not applicable.

[a]The genotypes and references for these strains can be found in the legends to Figures 13.4.1, 13.4.2, and 13.4.6, except for JA209 (*argH1 metE xyl trpA36 recA56 str^r*; Clarke and Carbon, 1978).

[b]Selection for α-aminoadipate resistance produces both *lys2*$^-$ and *lys5*$^-$ mutants. Selection for 5-FOA resistance generates both *ura3*$^-$ and *ura5*$^-$ mutants.

[c]This 1453-bp fragment contains both the *TRP1* and the *ARS1* genes.

[d]The wild-type *CAN1* gene encodes dominant sensitivity to the arginine analog canavanine sulfate.

[e]The sensitive (wild-type) and resistant alleles of the *CYH2* gene are codominant.

[f]The *CYH2* and *TCM1* genes encode ribosomal proteins, which are required for viability, but which can be mutated to confer resistance to cycloheximide or tricodermin, respectively.

[g]The resistant allele of *TCM1* is dominant to the wild-type sensitive allele.

on the plasmid and homologous sequences in the yeast genome. This recombination event results in a tandem duplication of the yeast sequences that bracket the rest of the plasmid DNA. If the YIp plasmid contains an incomplete portion of a cloned gene, this technique can be used to create a gene disruption (see *UNIT 13.10*). The reversal of the integration process occurs at a low frequency (~0.1% to 1% per generation), with excision of the integrated plasmid occurring by recombination between duplicated yeast sequences. The frequency of transformation of YIp plamids is only 1 to 10 transformants/μg DNA, but transformation frequency can be increased 10- to 1000-fold by linearizing the plasmid within yeast sequences that are homologous to the intended site of integration on the yeast chromosome. Linearization also directs the integration event to the site of the cleavage, which is useful when several different homologous yeast sequences are present on the plasmid.

Three classes of yeast vectors are circular plasmids capable of extrachromosomal replication in yeast. YRp plasmids (yeast replicating plasmids) contain sequences from the yeast genome which confer the ability to replicate autonomously. These autonomous replication sequences (*ARS*) have been shown to be chromosomal origins of replication. YRp plasmids have high frequencies of transformation (10^3 to 10^4 transformants/μg DNA), but transformants are very unstable both mitotically and meiotically. Despite the fact that *ARS*-containing plasmids replicate only once during the cell cy-

Leaders. As in other organisms, the translation efficiency in yeast is influenced by DNA sequences upstream of the gene coding sequence. However, the relationship between the sequence of untranslated mRNA leader sequences and translation efficiency in yeast is not very well understood, and a body of largely anecdotal evidence suggests that sequence requirements are not very restrictive. Typically, the only step taken to ensure proper translation is to make sure that the ATG that begins the gene's coding sequence encodes the first AUG in the mRNA. However, ATG fusion vectors (like those described in *UNIT 16.4*) containing untranslated sequences from well-expressed genes are now available in some systems (Table 13.5.1).

Terminators. By contrast, most yeast expression vectors do carry "transcription terminators" downstream from the promoter and the cloning sites. As their name implies, these sequences are thought to cause RNA polymerase II to cease transcribing. While this has not been completely established, it is clear that their presence in transcribed regions causes formation of 3′-ended, polyadenylated transcripts. More importantly, there is some evidence that the presence of transcription terminators downstream of a promoter increases the total amount of message (perhaps by stabilizing it) as well as the total amount of protein expression.

Useful protein moieties. Many expression vectors also carry, downstream of the promoter, DNA that contains an ATG and that encodes a useful protein sequence. Examples of such moieties include the following: signal sequences, to direct secretion of the expressed protein into the extracellular medium; nuclear localization sequences, to direct its transport into the nucleus; and epitope or other protein tags such as those from Myc, influenza virus hemaglutinin, or the *E. coli TrpE* gene product, to facilitate its immunological detection and purification. Recently, vectors have been described that allow the expression of the protein fused to a eukaryotic transcription activation region; such fusion proteins are used to detect and study protein-protein interactions.

Reference: Schneider and Guarente, 1991.

Contributors: Roger Brent and Kaaren Janssen

UNIT 13.6

Yeast Vectors and Expression Assays

BASIC PROTOCOL 1

LACZ FUSION VECTORS FOR STUDYING GENE REGULATION

Because of the ease and sensitivity of the β-galactosidase assay, yeast genes are often "tagged" with a functional portion of the *lacZ* gene, to monitor the regulation of expression of the yeast gene in question. These fusions are constructed such that the promoter region of the yeast gene—plus several amino acids from the N terminus of the protein encoded by this gene—is fused to the carboxy-terminal region of the *lacZ* gene, which encodes a protein fragment that still retains β-galactosidase activity. When constructing *lacZ* fusions, it is crucial that the translational reading frame across the fusion junction is maintained. A more detailed discussion of construction of in-frame fusions can be found in Guarente (1983).

The plasmid pLG670-Z (Fig. 13.6.1) can be used for constructing *lacZ* fusions (Guarente, 1983). Two other plasmids (pLG200 and pLG400)—containing different translational reading frames and/or different unique restriction sites at the 5′ end of the *lacZ* fragment—have also been constructed (Guarente et al., 1980). These two plasmids do not contain selectable yeast genes or yeast replication origins but can be used to first construct an in-frame fusion, which is then transferred onto a yeast shuttle vector (*UNIT 13.4*).

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 0378-1119/95/$09.50

GENE 08709

A double counter-selection system for the study of null alleles of essential genes in *Escherichia coli*

(SecE; suppressor mutation; *rpsL*; *lacY*; suicide plasmid)

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SUMMARY

The ability to analyze null alleles of genes can be an important means of studying both a protein's function and its interactions with other proteins involved in a particular process. However, if the gene encodes a protein that is essential to the viability of the cell, such analysis becomes complicated because a complementing copy of the gene must be present in the cell. In order to study the effects caused by the null allele, the complementing copy must be inactivated or lost. We report the development of a system in *Escherichia coli* which facilitates the manipulation of null alleles of essential genes. It consists of a strain deleted chromosomally for the essential gene and complemented for its function by a wild-type (wt) copy expressed from a plasmid counter-selectable for two markers bracketing the gene. Using this system, we have (*i*) searched for bypass suppressors of a deletion of the essential *secE* gene, (*ii*) ascertained the ability of various mutant *secE* genes to complement a deletion of the wt copy and (*iii*) isolated intragenic pseudorevertants of a null missense allele of *secE*. This methodology should be widely applicable to other cases in which essential genes are to be studied genetically.

INTRODUCTION

The genetic analysis of essential cellular processes has relied heavily on the use of conditional lethal mutants. Such mutants have allowed the detection of sets of indispensable genes involved in particular pathways and have provided a means of studying the physiological effects of depleting cells of a protein in the pathway. However, studies using conditional lethal alleles are limited for various reasons. First, the conditional lethal mutants most often used are ts mutants, which are likely to affect the folding and stability of a protein rather than alter aa most important to its function (including the active site residues). Thus, conditional mutations are unlikely to permit the identification of residues crucial to the function of a protein. Second, the mutant protein in conditional lethal mutants may still exhibit some function when subjected to the non-permissive condition. In such cases, the physiology of the cell under the non-permissive condition may not give an accurate picture of the physiology of a cell totally lacking the gene product. Finally, while suppressor analysis using conditional lethal alleles has resulted in the successful identification of interacting components within a pathway, the fact that such alleles are not totally null for function sometimes leads to the detection of suppressors that are only indirectly related to the process in

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Abbreviations: aa, amino acid(s); Ap, ampicillin; AP, alkaline phosphatase; Km, kanamycin; MSS, membrane-spanning segment; MTSS medium, minimal tONPG streptomycin succinate; oligo, oligodeoxyribonucleotide; PCR, polymerase chain reaction; ^{R}, resistance/resistant; ^{S}, sensitivity/sensitive; Sm, streptomycin; Tc, tetracycline; Tn, transposon; tONPG, 2-nitrophenyl-β-D-thiogalactoside; ts, temperature sensitive; wt, wild type; ::, novel junction (fusion or insertion).

SSDI 0378-1119(94)00920-1

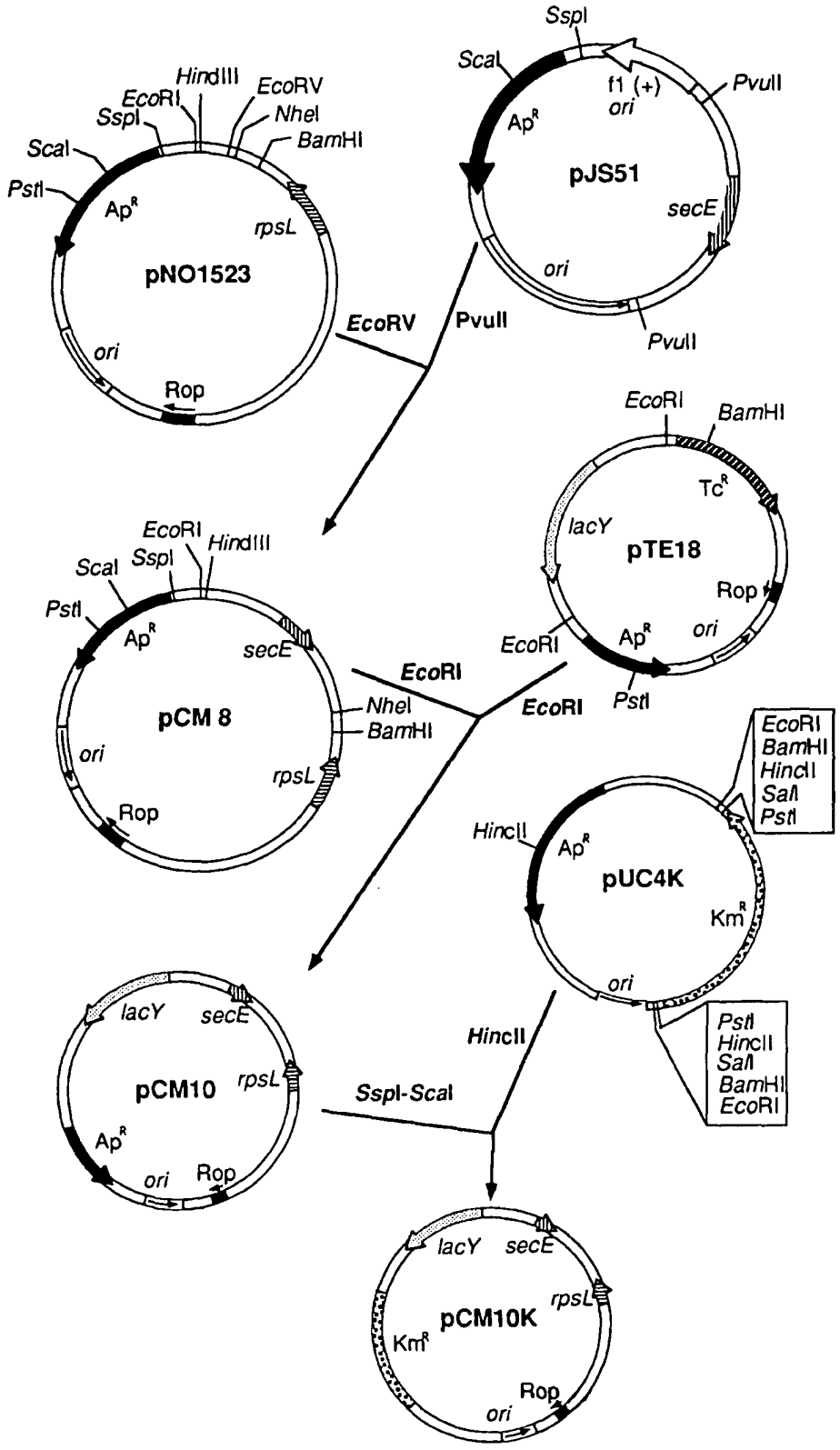

Fig. 1. Construction of counter-selectable plasmids. A general schematic for the construction of various plasmids described in this paper is presented. All DNA modification or restriction enzymes were purchased from New England Biolabs (Beverly, MA, USA). pCM8 was made by cloning the *secE*-containing *Pvu*II fragment from pJS51 into the *Eco*RV site of pNO1523. The latter plasmid is a pBR-derived plasmid (Dean, 1981) expressing the wt *rpsL* gene, and which confers dominant sensitivity to Sm (1.5 mg/ml). To construct pCM10, the *lacY*-containing *Eco*RI fragment from pTE18 (Teather et al., 1980; kindly provided by Dr. Tom Wilson) was cloned into the *Eco*RI site of pCM8. The *lacY* gene of the resulting plasmid confers

which the original protein is involved (e.g., Lee et al., 1986; Shiba et al., 1986).

An approach to obviating some of these problems is the construction of null mutations (e.g., extensive deletions). However, null mutations in essential genes can only be constructed when the cell carries an extra copy of that gene. To study the properties of cells lacking the essential gene product, the complementing copy may be expressed under the control of a regulatable promotor, thus allowing for the depletion of the essential gene product. Although such strains may reveal the null phenotype for an essential gene, they are not particularly useful for obtaining suppressor mutations. For instance, (*i*) selection for suppressors may yield a high frequency of mutations that affect the regulated promoter resulting in constitutive expression of the complementing copy of the essential gene and (*ii*) because the gene product is totally missing, suppressor analysis may not reveal components that interact with it.

We have addressed certain of the problems described above by developing a system in which a complementing copy of the essential gene of interest can be strongly selected against. The essential gene used in these studies, *secE*, encodes a member of the protein secretion apparatus of *Escherichia coli* (Schatz et al., 1989). In our system, *secE* is expressed from a plasmid in a strain deleted for its chromosomal copy. By bracketing the *secE* gene with two genes for which there are strong counter-selections we have facilitated screens for null missense mutants and the search for suppressors of such mutants. The basic approach reported here for the analysis of null alleles of *secE* should be applicable to the study of essential genes in other systems.

RESULTS AND DISCUSSION

(a) Construction of a doubly counter-selectable plasmid for *secE*: rationale

Genes which can be selected against, or so-called 'positive selection' genes, have been exploited for a number of genetic studies, including the construction of recombinant libraries and gene cloning (Arakawa et al., 1991; Cheng and Modrich, 1983), allelic exchange (Russel and Dahlquist, 1989; Quandt and Hynes, 1993; Skrzypek et al., 1993; Slater and Maurer, 1993), isolation of insertion sequences (Simon et al., 1991), and the counter-selection of organisms released into the environment (Recorbet et al., 1993). In all of these studies, cells harboring the counter-selectable genes, when exposed to the counter-selective agent, readily yield viable cells. However, a percentage of these viable cells are the result of undesired events leading to inactivation of the positive selection gene, such as deletion or point mutations within it. Often the percentage of these events is quite high, but does not detract from the results of the studies, because secondary screens are performed to insure that the expected strains are obtained.

The selections described above have utilized single counter-selectable markers that confer sensitivity to an agent (e.g., the *sacB* gene of *Bacillus sp.*, conferring sensitivity to sucrose (Gay et al., 1985), or *rpsL*, conferring sensitivity to Sm (Dean, 1981)). However we wished to use a counter-selectable plasmid in studies seeking rare suppressor mutations. Selecting against single, plasmid-encoded genes such as *sacB* or *rpsL* yields, in addition to cells which have lost the plasmid carrying them, a relatively high frequency of mutations that eliminate *sac* or *rpsL* function. This frequency could well be two or three orders of magnitude higher than the frequency of suppressor mutations, making the isolation of the suppressor mutants tedious, since a subsequent screen for loss of the plasmid would have to be performed. Therefore, we constructed a plasmid containing two counter-selectable genes (Fig. 1). Independent mutations occurring in both genes in the same plasmid should be extremely rare. Further, these genes bracket the gene under study (*secE*), since deletions within the plasmid could eliminate the counter-selectable genes in a single event, if the two genes lay on the same side of *secE*. Thus,

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sensitivity to 1 mM tONPG (Bachem, Torrence, CA, USA; Berman and Beckwith, 1979) on cells harboring it. pCM10K is a Km^R derivative of pCM10 made by cloning the Tn*903* aminoglycoside transferase gene (*aphA*) containing the *Hin*cII fragment from pUC4K (Vieira and Messing, 1982) into an *Ssp*I+*Sac*I-cut pCM10 that was treated with T4 DNA polymerase.

Strain PS291 {Schatz et al., 1991; *phoA*Δ*Pvu*II,*lac*ΔX74, *galE*, *galK*, *rpsL*150, *pcnB*80::*zadL*::Tn*10*(Sm^R, Tc^S), *recA*::*cat*, *secE*Δ19–111, pBRU (*secE*$^+$)} was used as a host strain for the counter-selectable plasmids for several reasons: (*i*) it contains a deletion of nearly the entire *secE* gene, including regions encoding all three MSS and the essential second cytoplasmic loop region; (*ii*) the *rpsL*150 allele makes the strain Sm^R; (*iii*) *lacY* is deleted, and therefore the strain is resistant to tONPG (Berman and Beckwith, 1979); (*iv*) the *pcnB*80 allele makes pBR-based plasmids harbored in this strain unstable, and reduces their overall copy number (Lopilato et al., 1986) In addition, the *pcnB*80 allele was important because *lacY* expressed from a high-copy-number plasmid is toxic to cells; strains that are wt for plasmid copy number tend to mutate the *lacY* gene to compensate for this toxicity; and (*v*) the *recA*::*cat* allele reduces the frequency of recombination in this strain. The strains used in this paper are complemented for the *secE* deletion by either of the counter-selectable plasmids in place of pBRU. In general, selection against the presence of the counter-selectable plasmids in this strain was carried out on MTSS plates (M63-minimal medium (Miller, 1992) containing 0.2% casamino acids/1 μ thiamine per ml/succinate (0.4%)/1.5 mg Sm per ml (Sigma, St. Louis, MO, USA)/1 mM tONPG (also previously described in Murphy and Beckwith, 1994). If entire cultures were plated on MTSS medium, they were subjected to one wash in M63 salts and plated at a density of 2×10^9 cells per plate. When appropriate, Ap (50 μg/ml) or Km (40 μg/ml) were included in the medium.

any cell surviving the double selection must have either lost the plasmid or have suffered a deletion which simultaneously deleted both genes plus *secE*. In either case, the selection would generate cells that had lost *secE*.

The *secE* gene was expressed from its own promoter on the counter-selectable plasmids, pCM10 or pCM10K (Fig. 1), which differ only in the antibiotic-resistance gene they carry. This plasmid-encoded *secE* is flanked by two counter-selectable genes, *rpsL* and *lacY*. The wt *rpsL* gene confers sensitivity to Sm and is dominant over the chromosomal *rpsL*150 Sm^R allele (Dean, 1981). The *lacY* gene encodes lactose permease, which confers sensitivity to the β-galactoside tONPG by allowing its entry into cells (Berman and Beckwith, 1979). Thus, this plasmid, and its wt copy of *secE*, can be strongly selected against simply by growing cells in the presence of Sm and tONPG (MTSS medium; Table I).

The strains used for genetic analyses involving the counter-selectable plasmid are derivatives of PS291, which contains a deletion (*secE*Δ19–111) of nearly the entire *secE* gene, including its three membrane-spanning segments (MSS; Schatz et al., 1991) and its essential cytoplasmic loop region (see legend to Table I; Murphy and Beckwith, 1994). Strains CM100 and CM263 are derivatives of PS291 complemented for *secE* by either pCM10 or pCM10K, respectively. These strains also carry (*i*) the *pcnB*80 allele, that results in reduced copy number and reduced stability of pBR-based plasmids (Lopilato et al., 1986) such as pCM10, (*ii*) *recA::cat*, that greatly reduces the frequency of recombination, (*iii*) *lac*X74 (deleted for the *lac* operon) and (*iv*) *rpsL*150 (Sm^R). Strains CM100 and CM263 do not grow on MTSS selective medium, indicating that loss of the $secE^+$ plasmid is fatal to the cell. These results provide more direct support for our previous conclusion (Schatz et al., 1991) that *secE* is an essential gene, as do SecE depletion experiments showing that the protein is essential for growth and secretion (B. Traxler and C.K.M., data not shown). To lose the counter-selectable *secE* plasmid and thus be viable on MTSS medium, CM100 or CM263 must be complemented in some other way for SecE activity.

TABLE I

Ability of mutations at codon 104 to complement *secE*Δ19–111

Codon(s)[a]	The aa^{104} change	Complementation of *secE*Δ19–111[b]
GGT, GGG, GGA	Gly	+
TGG	Trp	+
TTT	Phe	+
ATC	Ile	+
GCG, GCT	Ala	+
AAG, AAA	Lys	+ (ts)
CGG, AGG	Arg	−
GTG	Arg	+
TGT	Val	+
TTG	Cys	+
TAG, TAA	Stop codon	−
wt (ATG)[c]	Met	+
none (vector)[d]	No protein	−

[a] Randomization of codon 104 of *secE* was carried out by first PCR amplifying the *secE* gene using (*i*) an oligo containing the randomized codon of interest plus 15 nt corresponding to either side of the mutation and (*ii*) a downstream oligo that encompasses a unique restriction site in our $secE^+$ plasmids. These fragments were then gel-isolated and used as 'megaprimers' in a second PCR reaction (Sarkar and Sommer, 1990) with an oligo upstream of a second unique restriction site present in pJS51 ($secE^+$). These mutant fragments were then cloned in the place of the wt *secE* fragment in plasmid pJS51 using *Mlu*I and *Acc*65I. The collection of mutated plasmids was transformed into a wt *E. coli* strain, and random transformants were purified. The plasmid-encoded *secE* region was specifically amplified from the colonies by PCR and sequenced (as previously described in Murphy and Beckwith, 1994) to identify the mutation at aa^{104} in SecE.

[b] Complementation of the *secE*19–111 deletion was assessed by transforming purified *secE* mutant plasmids into CM263 (PS291 with pCM10K instead of pBRU; Murphy and Beckwith, 1994), and streaking the resultant Km^R and Ap^R colonies on MTSS plates. Plus (+) indicates that the strain grew within two days and subsequently became Km^s; minus (−) indicates failure to grow on MTSS. The Lys^{104} mutants did grow on MTSS and became Km^s but failed to grow at 42°C (ts).

[c] The wt *secE*-encoding plasmid pJS51 was transformed into CM263 as a control.

[d] Parent vector pBluescript KS+ (Stratagene, La Jolla, CA, USA) was used as a negative control.

(b) Selection for *secE* bypass mutations

We have previously shown that a small portion of the SecE protein is sufficient for its function (Schatz et al., 1991; Murphy and Beckwith, 1994), leading us to speculate that it might be possible to isolate *E. coli* mutants that bypass SecE function altogether. To isolate such bypass suppressors, CM100, carrying the *secE* deletion and the counter-selectable plasmid was incubated on MTSS medium over a period of two weeks. Colonies arose at a frequency of $(1–3) \times 10^{-8}$ per cell that tested as sensitive to Ap, indicating that they had lost pCM10 and were able to grow in the absence of the plasmid-encoded SecE. One hundred such independently isolated colonies were further analyzed by PCR for the presence of the *secE* gene, using both chromosome- and plasmid-specific oligos as described previously (Murphy and Beckwith, 1994). Although all of these colonies were found to be Ap^S, indicating that they had lost the counter-selectable plasmid, all had also retained a copy of the *secE* gene by recombination of the plasmid-encoded *secE* at either its chromosomal locus or elsewhere. These low-frequency events were occurring even though the host strain was $recA^-$. No bypass suppressor mutations were detected.

(c) The use f CM263 t test for the ability of mutations at Met[104] t c mplement for SecE function

One goal of our studies on SecE is to determine which regions or positions in the molecule are necessary for its function. We have previously performed both deletion and replacement mutagenesis of different portions of SecE (Beckwith and Murphy, 1994). In addition, we have generated point mutations at specific residues within these regions. This latter study has been carried out by the use of mutagenic oligos that randomize specific codons in the region encoding MSS3 of the protein. We have determined, by analyzing randomization of codon 104 (Met; Table I), that each mutagenesis experiment yields a collection of mutant plasmids in which the targeted codon is different from the wt codon nearly 100% of the time. A number of point mutations at codon 104 of *secE* were isolated, DNA sequenced, and tested for their ability to complement the chromosomal *secE*Δ19–111 deletion (Table I). The latter test was accomplished by asking whether the mutant plasmid could eliminate the requirement for the presence of the *secE*-complementing counter-selectable plasmid. Strain CM263 (*secE*Δ19–111, counter-selectable pCM10K (Km^R)) was transformed with each of the mutant plasmids to Ap resistance, and then restreaked on MTSS medium. Control strain CM263 transformed with plasmid pJS51 (pUC-based, $secE^+$; Schatz et al., 1989) gave rise to colonies that had lost pCM10K (and thus were able to grow on MTSS medium and were Km^S). In contrast, transformants harboring the non-*secE*-complementing parent vector pBlueScript failed to yield viable cells on MTSS medium, and these strains remained Km-resistant, indicating retention of pCM10K.

Of the mutations isolated, only those that introduced the charged aa Arg or Lys into position 104 of MSS3 were defective for SecE function, as evidenced by the inability of plasmids carrying these mutations to allow for the loss of pCM10K (Table I; the M104K mutation is ts). CM263 transformed with these plasmids remained $MTSS^S$ and Km^R. A number of other mutations at this position were able to complement for SecE function and allowed for the loss of pCM10K.

In addition to the detailed analysis of position 104 of MSS3, we have used CM263 to search for other positions in MSS3 that might be sensitive to mutation. Mutant plasmid libraries, corresponding to specific randomized positions of MSS3, were transformed into CM263, resulting in collections of Ap^R transformants that presumably contain every possible codon at each position. For each position in MSS3, 200 Ap^R colonies were randomly picked and restreaked on MTSS medium. The percentage of non-complementing plasmids (i.e., cells that were unable to yield colonies on MTSS) for each position was then calculated. In no case did this percentage exceed 10.5% (data not shown). This figure could represent the frequency of codons that result in drastic non-functional substitutions such as Arg residues (see section **d**) or stop codons. The inability to find a mutationally sensitive position in the MSS3 was not surprising since we have subsequently found that a total replacement of this MSS with a heterologous MSS results in a SecE hybrid able to complement for SecE function in CM263 (Murphy and Beckwith, 1994).

(d) Selection for suppressors of a non-functional missense allele of *secE*

One powerful application of the double counter-selection system is the ability to use it to select for suppressors of non-functional alleles of essential genes. We have carried out such a selection using as the starting non-functional missense allele of *secE*, the *secE*M104R mutation. We believe that the $SecE^-$ phenotype of this mutant is due to the alteration of the normal topology of SecE. Alkaline phosphatase (AP) fusions to this mutant form of the protein exhibit very low AP activity, indicating that the native topology of SecE has been disrupted, and that either MSS3 is not integrated into the cytoplasmic membrane, or that it is integrated in an unstable manner (Murphy and Beckwith, 1994).

To select for suppressors of the M104R mutation (Table II), a pACYC177 (Km^R) derivative, pCM68, carrying the mutant gene was transformed into CM100. The resulting strain, CM105, carries the missense mutation M104R of *secE*, the *secE*Δ19–111 chromosomal deletion,

TABLE II

Intragenic suppressors of the M104R mutation in SecE[a]

Codon/No. of times isolated	aa	Complementation of *secE*Δ19–111
ATG/7	Met	+
AAG/2	Lys	+ (ts)
AGC/1	Ser	+

[a] To facilitate isolation of intragenic suppressor mutations to the M104R mutation (Table I), the *Pvu*II fragment encoding this mutant form of SecE was cloned from pJS51M104R (Murphy and Beckwith, 1994; Table I) into pACYC177 that had been digested with *Fsp*I + *Hinc*II. The resulting plasmid is compatible with pBR-based plasmids, such as the counter-selectable plasmids, and confers resistance to Km (40 μg/ml). This plasmid was transformed into CM100 (PS291, pCM10(Ap^R) instead of pBRU) yielding strain CM105. Cultures of CM105 were plated on MTSS medium with added Km as described in the legend to Fig. 1. Colonies that arose were picked for a two-week period and were subsequently tested for Ap sensitivity to ascertain whether they had lost pCM10. Plasmid from these colonies was then isolated and retransformed into CM100. Ten independently isolated plasmids that allowed for growth of CM100 on MTSS plates were subjected to nt sequence analysis, as described in footnote a to Table I.

and is dependent for growth on complementation by the counter-selectable $secE^+$ copy on pCM10 (Ap^R). Mutant cells that could lose the counter-selectable plasmid and thus use the *secE* copy encoded by pCM68 were selected for by plating 5×10^{10} CM105 cells on MTSS medium with added Km. Colonies that arose over a two-week period were picked and subjected to further analysis. None of the 31 independent colonies screened further were Ap^R, indicating that they had probably lost the counter-selectable plasmid. Plasmid DNA from these colonies was isolated and retransformed into CM100, again selecting for the ability to complement the chromosomal deletion of *secE*. Of the 31 plasmids, 16 were able to complement the *secE* deletion by this criterion, suggesting that the suppressor mutations were intragenic. Ten of these independently isolated plasmids were subjected to nt sequence analysis. Seven plasmids had either reverted to the wt Met codon, or had recombined wt *secE* from the counter-selectable plasmid to the pACYC plasmid. One of the remaining three plasmids contained a Lys^{104} codon and two contained a Ser^{104} codon.

(e) Conclusions

These studies allow some estimates of the mutation frequencies we are looking at in studies with the counter-selectable plasmid. First, we point out that in the selection for mutants that bypass the requirement for SecE, colonies continue to appear on the plate for several weeks. As we have shown that these are all due to recombination of a wt *secE* gene from plasmid to chromosome, it means that revertants can arise from colonies still carrying the plasmid many days after the plating. At first glance, this appears surprising since the diploid for *rpsL* is sensitive to Sm, which is ordinarily a bacteriocidal agent. However, in such diploids, it has been established that Sm does stop cell growth, but is bacteriostatic (Breckenridge and Gorini, 1969). In fact, our data suggest that a high proportion of cells plated have the potential to survive if a mutational event that causes reversion occurs. The frequency of a single base substitution yielding a particular revertant of M104R is about 10^{-9}. This is comparable to mutation frequencies found for such substitutions in *E. coli*, in general. A qualification to this calculation is that even though the plasmid carrying M104R is present in relatively low copy number because of the *pcnB* mutation, the detectable mutation frequency may be higher than that for chromosomal mutations due to the increased copy number. At any rate, it is clear that we can detect very rare mutations using this system. The sensitivity of the selection system is relevant to our search for bypass suppressors of the *secE* deletion strain. We failed to find such suppressors among approx. 10^{10} cells. Thus, we consider it unlikely that it is possible to bypass the function of SecE by single mutational events.

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Activation of the *leu-500* Promoter by a Reversed Polarity *tetA* Gene

RESPONSE TO GLOBAL PLASMID SUPERCOILING*

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The *leu-500* promoter is inactivated by a mutation in the −10 region but can be activated in *topA Escherichia coli* and *Salmonella* strains. We have found that the *tetA* gene plays a vital role in the *topA*-dependent activation of a plasmid-borne *leu-500* promoter. In previous studies, the *leu-500* promoter and *tetA* gene have been arranged divergently. In this study we have reversed the polarity of the *tetA* gene, thus locating the *leu-500* promoter at the 3′ end of *tetA*. Despite being formally located in the downstream region of *tetA*, the *leu-500* promoter is equally well activated in a *topA* strain in this environment, even though it is 1.6 kilobase pairs away from the promoter of the reversed *tetA* gene. Activation of the *leu-500* promoter depends on transcription and translation of *tetA* but is largely insensitive to the function of other transcription units on the plasmid. These results require a change in viewpoint of the role of *tetA*, from local to global supercoiling. We conclude that transcription of the *tetA* gene is the main generator of transcription-induced supercoiling that activates the *leu-500* promoter. Unbalanced relaxation of this supercoiling leads to a net increase in the negative linking difference of the plasmid globally, and there is a linear correlation between the change in global plasmid topology and the activation of the *leu-500* promoter. Thus the *leu-500* promoter appears to respond to the negative supercoiling of the plasmid overall.

The activation of the *leu-500* promoter provides a good illustration of the possible interrelationships between transcription and the topology of the DNA template *in vivo*. *leu-500* is a leucine auxotroph of *Salmonella typhimurium* (1) that results from an A to G transition in the −10 region of the promoter of the *leu* biosynthetic operon (2). It was found that leucine prototrophy was restored in *Salmonella* bearing a *supX* mutation (3). The later demonstration that *supX* was identical to *topA* (4), the gene encoding DNA topoisomerase I, provided a strong indication of a functional link between transcription and topology. Thus the increase in negative supercoiling that should arise from the loss of the supercoiling-relaxation activity from the *Salmonella* cell (5) might be expected to assist in the function of the *leu-500* promoter, coupling the additional free energy of negative supercoiling to the opening of the more refractory −10 region of the mutant promoter (6, 7).

More recent work in this laboratory has identified an additional level of complexity in this process. The demonstration of a direct requirement for a null *topA* background (8) led to the suggestion that the *leu-500* promoter might be activated by variations in template supercoiling arising from transcriptional-induced supercoiling due to the transcription of a nearby gene (9, 10). According to the twin-supercoiled domain model of Liu and Wang (11), a rotationally hindered RNA polymerase in the elongation phase of transcription will tend to generate positive supercoiling ahead of its passage and negative supercoiling in its wake. These domains will be relaxed by DNA gyrase and topoisomerase I, respectively, in eubacteria, but unbalanced relaxation by topoisomerase activity due to either inhibition or mutation will lead to alteration in the local level of DNA supercoiling (12, 13). Thus the *leu-500* promoter might be activated by negative supercoiling arising from the transcription of the putative nearby gene, which would be less efficiently relaxed in *topA* cells.

Although this model could explain the activation of the chromosomal *leu-500* promoter in *topA Salmonella*, a further complication came to light when we sought to reproduce the effect on a plasmid. We found that we could only obtain significant activity of the *leu-500* promoter when the plasmid also bore the gene encoding resistance to tetracycline, *tetA*. Using such plasmids we could achieve *topA*-dependent activation of the promoter in either *Salmonella* (10) or *Escherichia coli* (14). This implied a key role for the *tetA* gene, and a number of studies have indicated that the coupled transcription, translation, and membrane insertion of the *tetA* gene product are essential for efficient oversupercoiling of plasmids in *topA* eubacterial cells (13, 15–17) due to the anchorage of the transcribing RNA polymerase to the membrane. We showed that activation of the *leu-500* promoter on a plasmid did indeed require transcription and translation of the *tetA* gene and insertion of the TetA polypeptide into the membrane (10, 18).

We can conceive of two roles for the *tetA* gene in the activation of the *leu-500* promoter on a plasmid. First, transcription of the *tetA* gene could be the primary generator of supercoiling; tethering RNA polymerase to the membrane would be a particularly effective way in which to hinder its rotation about the DNA template, and thus efficient induction of supercoiling might be expected. The second role could be more passive: to provide a barrier to the diffusion of supercoiling. If negative and positive domains of supercoiling were generated by transcription elsewhere on the plasmid, these could diffuse around the circle and cancel each other by rotation about the duplex axis, providing a highly efficient nonenzymatic relaxation mechanism. However a point of anchorage (such as the insertion of the nascent TetA polypeptide into the membrane) should provide a barrier to the diffusion of supercoiling around the plasmid and might thus increase local levels of DNA supercoiling.

In the plasmid pLEU500Tc, with which we first achieved the *topA*-dependent activation of the *leu-500* promoter (10), the

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TABLE I
List of plasmids used in this work

pLEU500Tc	Original plasmid containing *leu*-500 promoter and clockwise *tetA*
pL500TR	Fully functional anticlockwise *tetA*
pL500TR.$\Delta P_{tet.rev}$	pL500TR with deletion of anticlockwise *tetA* promoter
pL500TR.Tet48	pL500TR with translation terminator in *Nhe*I site of anticlockwise *tetA*
pL500TR.Tet96	pL500TR with translation terminator in *Bam*HI site of anticlockwise *tetA*
pL500TR.Tet188	pL500TR with translation terminator in *Sal*I site of anticlockwise *tetA*
pL500TR.Tet296	pL500TR with translation terminator in *Nru*I site of anticlockwise *tetA*
pL500TR.Δbla	pL500TR with 30% N-terminal deletion of *bla*
pL500TR.ΔP_{tet}	pL500TR with deletion of clockwise *tetA* promoter
pL500TR.Δbla ΔP_{tet}	pL500TR with deletions of *bla* and clockwise *tetA* promoter
pL500TR.ΔP_{tet} $\Delta P_{tet.rev}$	pL500TR with deletion of clockwise and anticlockwise *tetA* promoters
pL500TR.Bla12	pL500TR with translation terminator in *Eco*57 site of *bla*
pL500TR.Bla80	pL500TR with translation terminator in *Sca*I site of *bla*

tetA gene was oriented divergently to the *leu-500* promoter, with a short distance between the promoters. This places the *leu-500* promoter immediately upstream of the *tetA* promoter, which would be consistent with a very local effect whereby the *leu-500* promoter responds to a domain of negative supercoiling directly upstream of *tetA*. We therefore wondered if the *leu-500* promoter would still be activated in *topA* cells if the orientation of the *tetA* gene were reversed. We find that the *leu-500* promoter is activated to the same level under these circumstances and that the activity remains fully dependent on the function of the *tetA* gene. We conclude that transcription of the *tetA* gene is the major source of negative supercoiling that activates the *leu-500* promoter, but that this is mediated through the global topology of the plasmid.

MATERIALS AND METHODS

Bacterial Strains and Their Growth Conditions

E. coli strains HB101 (F^-, *hsdS20* (r^-B, m^-B), *recA13*, *ara-14*, *proA2*, *lacYI*, *galK2*, *rpsL20* (Sm^r), *xyl-5*, *mtl-1*, *supE44*, λ^-), and DM800 *(Δ(topA-cysB)204 acrA13 gyrB225)* (25, 26) have been used in the experiments reported here. Bacteria were cultured at 37 °C with aeration in LB medium or grown on 1.2% LB agar plates. Media were supplemented with antibiotics as required; ampicillin and kanamycin were both used at 50 μg/ml and tetracycline was used at 10 μg/ml (except for strains related to *E. coli* DM800, where this was reduced to 2 μg/ml tetracycline). *E. coli* strains were transformed with plasmids using the calcium chloride procedure (27).

Plasmid Constructions

The plasmids used in this work are summarized in Table I

pL500TR—The plasmid pLEU500Tc (10) was cleaved with *Nhe*I and *Bal*I, and pAT153 (28) was digested with *Eco*RI and *Bal*I. The *Nhe*I and *Eco*RI termini were rendered flush by incubation with 2.5 units of VentR DNA polymerase (NEB) at 72 °C for 30 min. The smaller *Eco*RI-*Bal*I fragment of pAT153, containing the entire *tetA* gene, and the larger *Nhe*I-*Bal*I fragment of plasmid pLEU500Tc were isolated by preparative gel electrophoresis. The two blunt-ended fragments were then ligated together with T4 DNA ligase, and the resulting plasmid was transformed into *E. coli* HB101. The plasmid containing the complete *tetA* gene oriented anticlockwise (see Fig. 1) was identified by restriction digestion of isolated plasmid DNA.

pL500TR.$\Delta P_{tet.rev}$—pL500TR was cleaved with *Cla*I, and the resulting linear DNA was digested with mung bean nuclease (35 units/μl for 25 min at 37 °C). The blunt-ended DNA was religated to generate a plasmid that contained the modified anticlockwise *tetA* promoter.

pL500TR.ΔP_{tet}—To remove the −10 region of the original (clockwise) *tetA* promoter, pL500TR was partially cleaved with *Hin*dIII followed by digestion with mung bean nuclease (35 units/μl for 25 min at 37 °C). The blunt-ended DNA was religated and transformed into *E. coli* HB101. Since the *Hin*dIII cleavage could occur at either of the target sites, restriction digests and DNA sequencing were used to identify the deletion of clockwise-oriented *tetA* promoter.

pL500TR.ΔP_{tet} $\Delta P_{tet.rev}$—This plasmid contains deletions of both *tet* promoters of pL500TR. It contains a 4-bp[1] deletion at the *Hin*dIII site overlapping the clockwise-oriented *tetA* promoter and a 4-bp deletion at the *Cla*I site of the anticlockwise-oriented *tetA* promoter.

pL500TR.Δbla—This plasmid contains a deletion between the *Ssp*I site and the *Sca*I site of the *bla* gene. pL500TR was cleaved at the *Ssp*I and *Sca*I sites, the largest blunt-ended fragment was isolated by preparative electrophoresis in an agarose gel, and circularized with T4 DNA ligase.

pL500TR.Δbla ΔP_{tet}—This plasmid combines the deletion between the *Ssp*I site and the *Sca*I site of the *bla* gene and the 4-bp deletion at the *Hin*dIII site of the clockwise-oriented *tetA* promoter in pL500TR.

Plasmids Containing Translation Terminators within the tetA Gene of pL500TR—Pairs of complementary oligonucleotides (10) were ligated into the plasmid pL500TR linearized by the appropriate restriction enzyme; *Nhe*I (partial digestion was required since there are two *Nhe*I sites in pL500TR), *Bam*HI, *Sal*I, and *Nru*I, generating pL500TR.Tet48, pL500TR.Tet96, pL500TR.Tet188, and pL500TR.Tet296, respectively.

pL500TR.Bla12, pL500TR.Bla80—These plasmids contain translation termination codons inserted into either the *Eco*57 or *Sca*I sites within the *bla* gene of pL500TR. Self-complementary oligonucleotides encoding a universal translation terminator (18) were inserted into the *Eco*57 or the *Sca*I sites within the *bla* gene of pL500TR, generating pL500TR.Bla12 and pL500TR.Bla80, respectively.

Extraction and Analysis of Cellular RNA

RNA was isolated using essentially the method described previously (10). RNA was prepared from 200-μl cultures in the mid-exponential growth phase by the addition of an equal volume of 20 mM sodium acetate (pH 5.2), 2% SDS, 0.3 M sucrose and transferring to a boiling water bath for 1 min. The sample was then extracted twice with phenol/chloroform, and the nucleic acids were precipitated with ethanol. After the addition of 0.2 pmol of the appropriate radioactively [5′-^{32}P]-labeled DNA primer, the sample was heated to 90 °C in 4.5 μl of 50 mM Tris-HCl (pH 8.0), 50 mM KCl, and rapidly cooled. 25 units of RNasin (0.5 μl) were added, and the solution was incubated at 43 °C for 20 min before the addition to 12 μl of 70 mM Tris-HCl (pH 8.0), 70 mM KCl, 15 mM $MgCl_2$, 15 mM dithiothreitol, 1.3 mM deoxynucleoside triphosphate mixture containing 50 units of moloney murine leukemia virus reverse transcriptase (Superscript Plus; Life Technologies, Inc.) and incubated at 42 °C for 2 h. cDNA transcripts were electrophoresed in 6% polyacrylamide gels in 90 mM Tris borate (pH 8.3), 10 mM EDTA (TBE) containing 7 M urea, next to sequence markers generated by dideoxy sequence reactions (29) using the same primer. After drying the gels, radioactive fragments were visualized by autoradiography at −70 °C with intensifier screens or with storage phosphor screens and a 400 S PhosphorImager (Molecular Dynamics). Quantitation of radioactivity was performed directly upon the phosphorimage using ImageQuant (Molecular Dynamics).

Analysis of Linking Number of Extracted Plasmid DNA—E. coli cells were grown in 30 ml of LB plus appropriate antibiotics to mid-exponential growth phase, and the plasmid DNA was extracted using the Wizard Plus DNA extraction system (Promega). The purified DNA was electrophoresed in 1% agarose gels in TBE containing 2 μg/ml chloroquine. After electrophoresis, the gels were subjected to extensive washing in water followed by staining in 1 μg/ml ethidium bromide and further washing in water. The stained gels were photographed under UV illumination with red and green filters to remove background fluorescence. The photographic negatives were scanned electronically, and a negative image was presented.

RESULTS

Reversal of the tetA Gene of pLEU500Tc—In previous studies we showed that the activation of the *leu-500* promoter on the

[1] The abbreviation used is: bp, base pair(s).

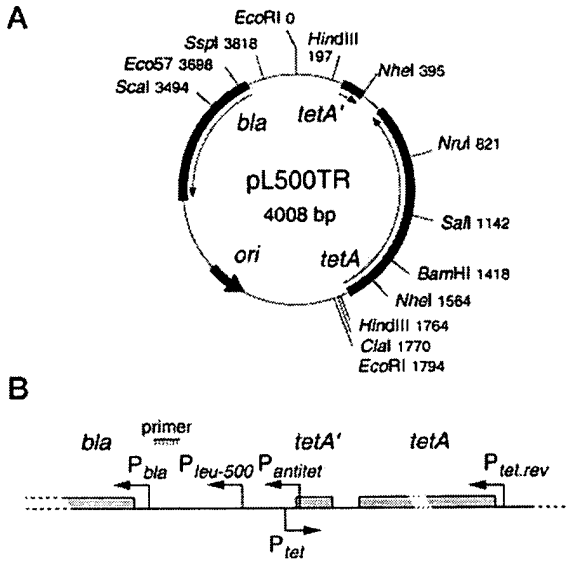

FIG. 1. **Map of pL500TR; a plasmid carrying the *leu-500* promoter and a reversed polarity *tetA* gene.** *A*, circular map of pL500TR showing the locations of the principal open reading frames, the replication origin (*ori*), and the locations of restriction sites. The original *tetA* gene from pLEU500Tc is truncated at the 48th codon and is designated *tetA'*. *B*, expansion of the region from *bla* through to the reversed *tetA* gene, showing the locations of the promoters. The position of the primer used in the reverse transcription analysis of transcripts is indicated.

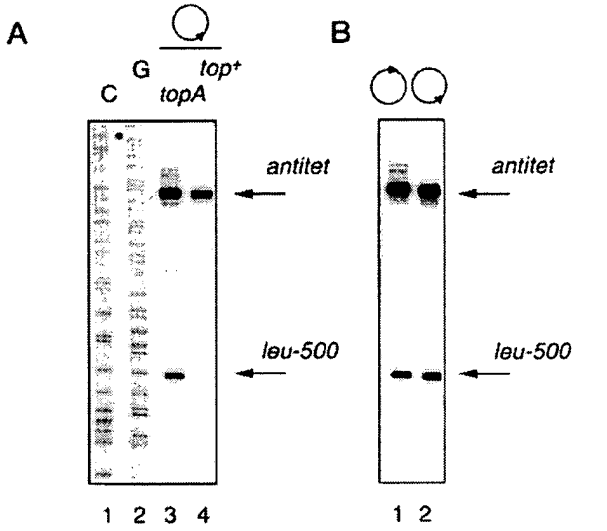

FIG. 2. **The *leu-500* promoter is activated in *topA E. coli* on a plasmid carrying a reversed polarity *tetA* gene.** *A*, activation of the *leu-500* promoter on pL500TR in *topA* and *top*$^+$ cells. mRNA was extracted from *E. coli* DM800 (Δ*topA*, *lane 3*) and SD108 (*top*$^+$, *lane 4*) and subjected to reverse transcription. cDNA transcripts were analyzed by gel electrophoresis and autoradiography. *Lanes 1* and *2* contain sequence markers generated by C and G dideoxy sequencing reactions, respectively. The positions of cDNA species corresponding to initiation at the *antitet* and *leu-500* promoters are indicated on the *right*. *B*, activation of the *leu-500* promoter as a function of *tetA* polarity. mRNA was extracted from *E. coli* DM800 (Δ*topA*) transformed with either pLEU500Tc (clockwise *tetA* gene, *lane 1*) or pL500TR (anticlockwise *tetA* gene, *lane 2*). As in *A*, initiation of transcription at the *antitet* and *leu-500* promoters was analyzed by reverse transcription and electrophoretic separation of cDNA products.

plasmid pLEU500Tc in *topA S. typhimurium* was dependent on the function of the adjacent tetracycline resistance gene *tetA* (10). The orientation of the *tetA* gene in pLEU500Tc is opposite to that of the *leu-500* promoter, *i.e.* the *leu-500* promoter is located immediately upstream of the *tetA* gene. Thus transcription of *tetA* might be the major generator of negative supercoiling in this local region, by the mechanism of Liu and Wang (11). Activation of the *leu-500* promoter in pLEU500Tc required the coupled transcription and translation of *tetA* and the membrane insertion of its product (10, 18). This suggested that membrane insertion of the TetA protein was essential to provide an anchorage point, which might act as a topological barrier against the diffusion of DNA supercoiling. These two related yet distinct roles for the *tetA* gene might be dissected if its polarity were reversed in the plasmid, and we therefore constructed a new plasmid pL500TR that contains a *tetA* gene oriented anticlockwise in the conventional depiction of pBR322-based plasmids. The reversed *tetA* gene is fully functional, and transformed cells have normal levels of resistance to tetracycline. pL500TR still contains the original clockwise *tetA* promoter, but the gene (*tetA'*) is truncated at the 48th codon. It also contains the anticlockwise *antitet* promoter. The plasmid map of pL500TR is shown in Fig. 1.

topA-dependent Activation of the leu-500 Promoter of pL500TR—In our earlier study, we demonstrated *topA*-dependent activation of the *leu-500* promoter carried on plasmid pLEU500Tc containing a clockwise *tetA* gene. To investigate the effect of a reversed polarity *tetA* gene on the activity of the *leu-500* promoter, RNA was isolated from pL500TR-carrying *topA* or *top*$^+$ *E. coli* cells in mid-exponential growth, and transcripts initiated at the *leu-500* promoter were sought. This was achieved by means of run-off reverse transcription using a primer that hybridizes to the vector sequence upstream of the *S. typhimurium* DNA (10). A cDNA corresponding to RNA initiated at the *leu-500* promoter should be 191 nuceotides in length. Since the *antitet* promoter (the *tetR* promoter transcribing the same strand as the *leu-500* promoter) is retained on pL500TR, cDNA corresponding to initiation at this promoter would be 281 nucleotides in length and provides a useful reference for quantitation.

The results of the reverse transcription analysis are shown in Fig. 2*A*. There is a clear band of cDNA corresponding to initiation at the *leu-500* promoter in DM800 (Δ*topA*) cells, but the intensity of this species is very much lower for the RNA extracted from SD108 (*top*$^+$). The cDNA band corresponding to initiation at the *antitet* promoter is of similar intensity in both *top*$^+$ and Δ*topA* experiments. Thus the *leu-500* promoter was activated by the reversed polarity *tetA* gene, and this activation was dependent on the Δ*topA* background.

The activity of the *leu-500* promoter as a function of the polarity of the *tetA* gene is directly compared in Fig. 2*B* using cells carrying either pLEU500Tc or pL500TR. RNA was extracted from *E. coli* DM800 (Δ*topA*) in exponential growth and subjected to reverse transcription analysis as before. The level of initiation at the *leu-500* promoter is closely similar in both plasmids. Thus the *topA*-dependent activation of the *leu-500* promoter does not depend on the orientation of the *tetA* gene.

Activation of the leu-500 Promoter Requires Transcription of the Reversed tetA Gene—In its original orientation in pLEU500Tc, the *tetA* gene must be transcribed to activate the *leu-500* promoter (10). We therefore investigated whether this was also required when *tetA* was reversed in pL500TR. Plasmid pL500TR.Δ$P_{tet.rev}$ was constructed containing a 4-bp deletion in the *Cla*I site upstream of the reversed *tetA* promoter (anticlockwise). Cells containing the modified plasmid are sensitive to tetracycline, demonstrating the inactivation of the *tetA* gene. Cellular RNA was extracted from DM800 (Δ*topA*) harboring pL500TR or pL500TR.Δ$P_{tet.rev}$ and analyzed by reverse transcription as before. The results (Fig. 3) show that initiation of transcription at the *leu-500* promoter in pL500TR was signifi-

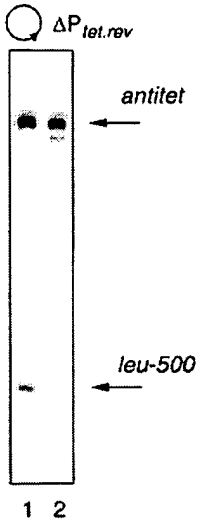

FIG. 3. **Effect of deletion of the promoter of the reversed polarity *tetA* gene on the activity of the *leu-500* promoter.** pL500TR.$\Delta P_{tet.rev}$ contains a deletion of the promoter of the reversed polarity *tetA* gene. It was transformed into *E. coli* DM800 (Δ*topA*), and RNA was isolated from cells in exponential growth. Initiation of transcription at the *antitet* and *leu-500* promoters was analyzed by reverse transcription and electrophoretic separation of cDNA products. The positions of cDNA species corresponding to mRNA initiated at the *antitet* and *leu-500* promoters are indicated on the *right*. *Lane 1*, RNA isolated from cells carrying pL500TR; *lane 2*, RNA isolated from cells carrying pL500TR.$\Delta P_{tet.rev}$. Note that inactivation of transcription of the reversed *tetA* gene leads to a marked reduction in activity of the *leu-500* promoter.

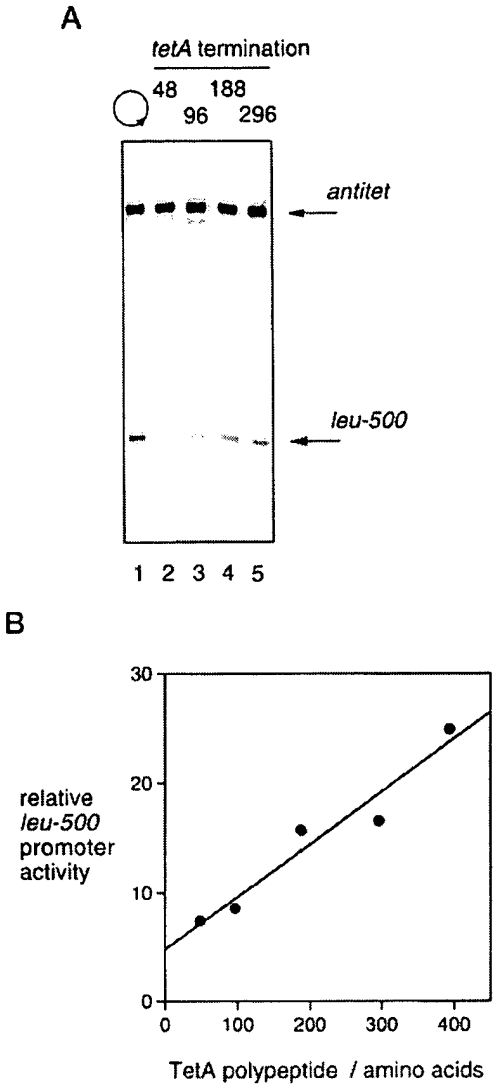

FIG. 4. **Effect of translation of the reversed polarity *tetA* gene on the activity of the *leu-500* promoter.** *A*, premature termination of translation of TetA was achieved by introducing termination codons at various positions along the gene, truncating the length of TetA polypeptide from 394 amino acids to 296, 188, 96, or 48 amino acids (plasmids pL500TR.Tet296, pL500TR.Tet188, pL500TR.Tet96, and pL500TR.Tet48, respectively). These were transformed into *E. coli* DM800 (Δ*topA*) and RNA isolated from cells in exponential growth. Initiation of transcription at the *antitet* and *leu-500* promoters was analyzed by reverse transcription and electrophoretic separation of cDNA products. The positions of cDNA species corresponding to mRNA initiated at the *antitet* and *leu-500* promoters are indicated on the *right*. *Lane 1*, pL500TR; *lane 2*, pL500TR.Tet48; *lane 3*, pL500TR.Tet96; *lane 4*, pL500TR.Tet188; *lane 5*, pL500TR.Tet296. *B*, plot of relative *leu-500* promoter activity as a function of the length of TetA polypeptide synthesized. The *line* was generated by regression, showing the linear correlation between the *topA*-dependent activation of the *leu-500* promoter and translation of the reversed *tetA* gene.

cantly reduced by the promoter deletion in the reversed *tetA* promoter. Thus the *topA*-dependent activation of the *leu-500* promoter in pL500TR requires transcription of the reversed *tetA* gene.

Activation of the leu-500 Promoter Requires Translation of the Reversed tetA Gene—By analogy with the role of the clockwise *tetA* gene of pLEU500Tc, it seemed probable that translation would be required in the reversed gene of pL500TR. This was examined by provoking premature termination of translation of the reversed *tetA* gene by introducing translation terminators at various positions in the coding sequence. This was achieved by introducing complementary oligonucleotides into the *Nhe*I, *Bam*HI, *Sal*I, and *Nru*I restriction sites along the *tetA* gene, thereby generating truncated TetA polypeptides of 48, 96, 188, and 296 amino acids, respectively. These can be compared with the full-length TetA that is 394 amino acids in length. These plasmids are called pL500TR.Tet48, pL500TR.Tet96, pL500TR.Tet188, and pL500TR.Tet296, respectively.

These plasmids were transformed into *E. coli* DM800 (Δ*topA*), RNA was prepared from cells in exponential growth, and the initiation of transcription from the *leu-500* promoter was analyzed by reverse transcription as before. Electrophoretic analysis of the cDNA (Fig. 4*A*) showed that the level of activity of the *leu-500* promoter became lower as the length of the translated reversed TetA polypeptide was reduced. The data were quantified by phosphorimaging and are presented graphically in Fig. 4*B*. Evidently the function of the *leu-500* promoter is dependent on translation of the reversed *tetA* gene, and the level of the activation of the *leu-500* promoter is approximately linearly dependent on the size of the TetA polypeptide synthesized. Thus the *topA*-dependent activation of the *leu-500* promoter depends both on transcription and translation of the reversed *tetA* gene. This closely parallels the situation where the *tetA* gene was oriented clockwise in the original construct pLEU500Tc, suggesting that a similar mechanism of activation of the *leu-500* promoter is involved in both cases.

Negative Supercoiling of Reversed-tetA Plasmids Isolated from ΔtopA E. coli—When plasmids carrying a functioning *tetA* gene are isolated from *topA E. coli* or *S. typhimurium* in exponential growth and their linking number distribution examined by electrophoresis in agarose gels containing the intercalator chloroquine, it is generally observed that there is a bimodal distribution of topoisomers, one fraction of which is very highly negatively supercoiled. We have previously shown this to be the case for pLEU500Tc and demonstrated a corre-

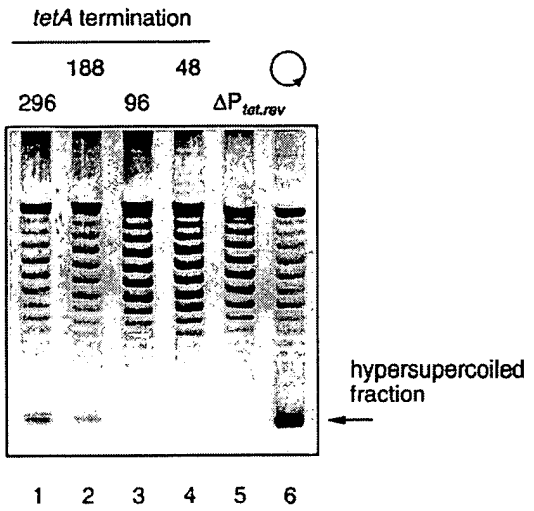

FIG. 5. **Topoisomer distributions of pL500TR and derivatives extracted from *topA E. coli*.** Plasmid DNA was isolated from DM800 in exponential growth, and topoisomers were separated by electrophoresis in agarose in the presence of chloroquine. pL500TR (*lane 6*) exhibits a typical bimodal distribution of topoisomers, with a hypersupercoiled fraction of DNA (indicated at the *right*). This was compared with the plasmids containing the translation terminators at various positions in the reversed *tetA* gene and with the plasmid containing the deletion of the promoter of the reversed *tetA* gene. The DNA was visualized by staining in ethidium bromide and photographed under UV light. A negative image is presented. *Lane 1*, pL500TR.Tet296; *lane 2*, pL500TR.Tet188; *lane 3*, pL500TR.Tet96; *lane 4*, pL500TR.Tet48; *lane 5*, pL500TR.$\Delta P_{tet.rev}$; *lane 6*, pL500TR.

lation between the degree of activation of the *leu-500* promoter and the extent of this hypersupercoiled fraction (14). We therefore examined the plasmids carrying the reversed *tetA* gene to see if these were similarly subject to hypersupercoiling.

Plasmid DNA was isolated from *E. coli* DM800 (Δ*topA*) in exponential growth and analyzed by electrophoresis in 1% agarose in TBE buffer containing 2 μg/ml chloroquine (Fig. 5). The distribution of pL500TR topoisomers was clearly bimodal, with a significant fraction of hypersupercoiled DNA. Reversing the polarity of the *tetA* gene has not changed its effect on the overall topology of the plasmid. Interference with the function of the *tetA* gene reduces the extent of this fraction of highly supercoiled plasmid. The proportion was severely reduced for pL500TR.$\Delta P_{tet.rev}$ (the plasmid containing a 4-bp deletion in the promoter of the reversed *tetA* gene), demonstrating the role of transcription of the reversed *tetA* in generating hypersupercoiled DNA. Translation of the *tetA* gene is also important for the hypersupercoiling, since the fraction of hypersupercoiled DNA was reduced in the plasmids where the *tetA* gene was interrupted by translation terminators; the shorter the translated TetA polypeptide, the smaller the fraction of hypersupercoiled DNA. Overall, there was a reasonable correlation between the fraction of hypersupercoiled DNA and the activity of the *leu-500* promoter for the different plasmid constructs containing a reversed *tetA* gene (see "Discussion").

Local Gene Expression and the Activation of the leu-500 Promoter in pL500TR—Analysis of the *topA*-dependent activation of the *leu-500* promoter in pL500TR clearly highlights the importance of the reversed *tetA* gene. We discussed two conceivable roles for this gene: as a direct generator of negative supercoiling by transcription with hindered rotation of RNA polymerase and as a topological barrier against the diffusion of negative supercoiling. Since the promoter of the *tetA* gene is a significant distance from the *leu-500* promoter in pL500TR, it is possible that the primary role is the latter function and that other more local promoters are important in the generation of supercoiling. We therefore turned our attention to other gene expression occurring within the vicinity of the *leu-500* promoter. This arises primarily from the *bla* gene and the original *tetA* gene of which the promoter is retained in pL500TR.

To determine the effect of local gene expression on the activity of the *leu-500* promoter, a number of new plasmids were constructed. pL500TR.Δ*bla* contains a 30% deletion in the *bla* gene, generated by removing the fragment between the *Ssp*I site and the *Sca*I site of the *bla* gene in pL500TR. The *bla* promoter is not directly affected by this deletion. pL500TR.ΔP_{tet} contains a 4-bp deletion in the *Hin*dIII site at the clockwise *tetA* promoter in pL500TR. This deletion is known to inactivate the promoter of the *tetA* gene (10). pL500TR.Δ*bla*ΔP_{tet} combines the deletions in the *bla* gene and in the clockwise-oriented *tetA* promoter. pL500TR.$\Delta P_{tet.rev}$ contains a 4-bp deletion at the *Cla*I site at the anticlockwise-oriented *tetA* promoter; this plasmid has been discussed above. pL500TR.$\Delta P_{tet}\Delta P_{tet.rev}$ contains both the deletion at the *Hin*dIII site at the clockwise-oriented *tetA* promoter and the deletion at the *Cla*I site at the anticlockwise-oriented *tetA* promoter.

These plasmids were transformed into *E. coli* DM800 (Δ*topA*), cellular RNA was isolated from cells in mid-exponential growth, and the initiation of transcription at the *leu-500* promoter was analyzed by primer extension as before (Fig. 6*A*). The activity of the *leu-500* promoter in pL500TR.Δ*bla* (*lane 4*) was not significantly less than that in pL500TR, indicating that *bla* was not important in the *topA*-dependent activation of the *leu-500* promoter. Deletion of the clockwise-oriented *tetA* promoter that remains in pL500TR (pL500TR.ΔP_{tet}; *lane 5*) also had very little effect on the activity of the *leu-500* promoter. Even the combination of both *bla* and clockwise *tetA* promoter deletions (pL500TR.Δ*bla* ΔP_{tet}; *lane 6*) resulted in a relatively minor reduction in *leu-500* promoter activity.

By contrast, as we have seen above, deletion of the promoter of the reversed *tetA* gene (*lane 7*) results in a marked reduction in activity of the *leu-500* promoter, and combination of the deletions of both *tetA* promoters results in a very similar low level of *leu-500* promoter activity (*lane 8*). We conclude that the dominant effector of the *topA*-dependent activation of the *leu-500* promoter is the reversed *tetA* gene.

Effect of Premature Termination of Translation of the bla Gene on the Activation of the leu-500 Promoter in pL500TR—Previous experiments showed that in the original construct with a clockwise *tetA* gene (pLEU500Tc), initiation of transcription at the *leu-500* promoter was influenced by translation of the *bla* gene under some circumstances (18). We therefore examined the effect of modulating the function of the *bla* gene on the activation of the *leu-500* promoter in the presence of the reversed *tetA* gene. Two new plasmids were constructed to examine the influence of *bla* translation. pL500TR.Bla12 and pL500TR.Bla80 contain translation termination codons inserted into the *bla* coding sequences at the *Eco*57 and the *Sca*I sites, respectively, generating β-lactamase polypeptides shortened from 263 amino acids to 12 or 80 amino acids, respectively.

The plasmids were transformed into DM800 (Δ*topA*), cellular RNA was isolated from cells in mid-exponential growth, and the initiation of transcription at the *leu-500* promoter was analyzed by reverse transcription as before (Fig. 6*B*). The activity of the *leu-500* promoter was not significantly reduced in either of these plasmids, indicating that translation of the *bla* gene is relatively unimportant in the activation of the *leu-500* promoter in pL500TR.

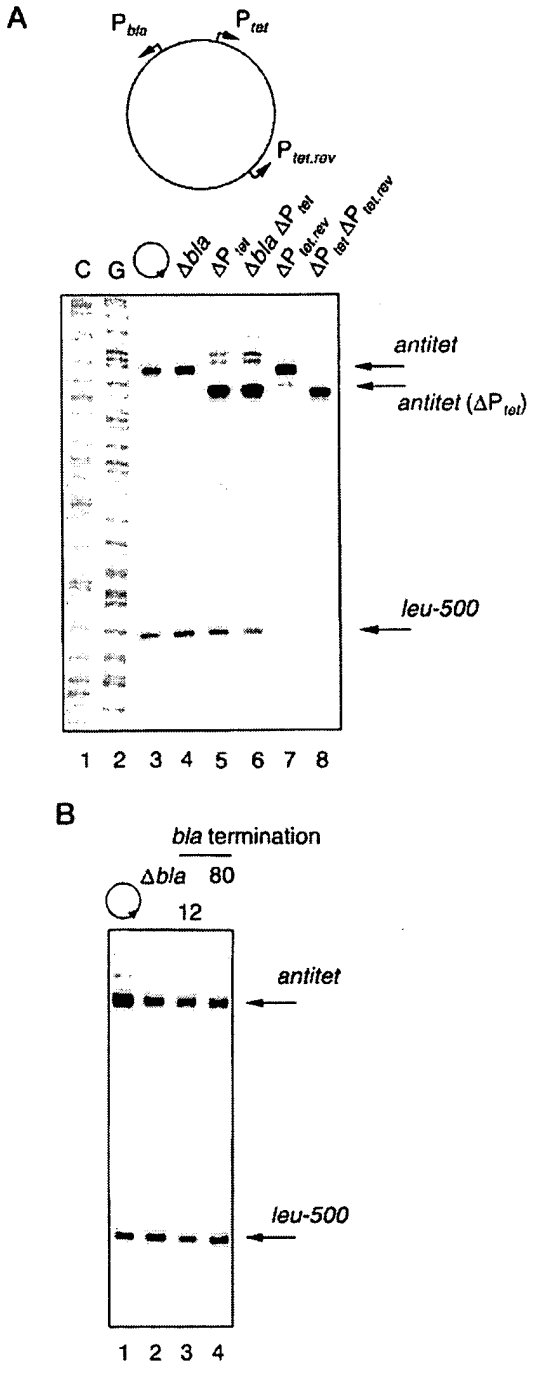

FIG. 6. **Effect of local gene expression on the activity of the *leu-500* promoter.** *A*, effect of local gene activity of the *leu-500* promoter. Deletions within the *bla* gene and the promoters of the truncated *tetA'* gene and reversed *tetA* gene of pL500TR were made in various combinations. These were transformed into *E. coli* DM800 (Δ*topA*) and RNA isolated from cells in exponential growth. Initiation of transcription at the *antitet* and *leu-500* promoters was analyzed by reverse transcription and electrophoretic separation of cDNA products. The positions of cDNA species corresponding to mRNA initiated at the *antitet* and *leu-500* promoters are indicated on the *right*. Note that the cDNA product corresponding to initiation at the *antitet* promoter in the ΔP_{tet} constructs is shortened due to the deletion introduced into the template. *Lanes 1* and *2* contain sequence markers generated by C and G dideoxy sequencing reactions, respectively. *Lane 3*, RNA isolated from cells carrying pL500TR; *lane 4*, RNA isolated from cells carrying pL500TR.Δ*bla*; *lane 5*, RNA isolated from cells carrying pL500TR.ΔP_{tet}; *lane 6*, RNA isolated from cells carrying pL500TR.Δ*bla* ΔP_{tet}; *lane 7*, RNA isolated from cells carrying pL500TR.$\Delta P_{tet.rev}$; *lane 8*, RNA isolated from cells carrying pL500TR.ΔP_{tet} $\Delta P_{tet.rev}$. *B*, effect of β-lactamase translation on the activity of the *leu-500* promoter. Translation termination codons were inserted into the *bla* gene of pL500TR so as to reduce the length of β-lactamase polypeptide to 12 or 80 amino acids. These plasmids were transformed into *E. coli* DM800 (Δ*topA*) and RNA isolated from cells in exponential growth. Initiation of transcription at the *antitet* and *leu-500* promoters was analyzed by reverse transcription and electrophoretic separation of cDNA products. The positions of cDNA species corresponding to mRNA initiated at the *antitet* and *leu-500* promoters are indicated on the *right*. *Lane 1*, RNA isolated from cells carrying pL500TR; *lane 2*, RNA isolated from cells carrying pL500TR.Δ*bla*; *lane 3*, RNA isolated from cells carrying pL500TR.Bla12; *lane 4*, RNA isolated from cells carrying pL500TR.Bla80.

DISCUSSION

Our results clearly demonstrate that the *leu-500* promoter can be activated on a plasmid in *topA E. coli* by the presence of a *tetA* gene in either orientation. Activation requires the full function of the *tetA* gene, but the *leu-500* promoter can be located in a position that can be regarded either as primarily upstream or one that is downstream of this gene. Moreover the role of the *tetA* gene is paramount; although other promoters present in pL500TR are of relatively minor consequence, inactivation of *tetA* function reduces the activity of the *leu-500* promoter to background levels. In summary, the *tetA* gene is essential for the *topA*-dependent activation of the *leu-500* promoter, but its orientation is unimportant.

It might be regarded as surprising that this effect is independent of *tetA* orientation; that the activation of the *leu-500* promoter is equally efficient when it is placed in what is formally the domain of positive supercoiling (downstream of *tetA*) (11), as when it is located in the upstream domain of negative supercoiling. We therefore change our perspective from a local view of variation in superhelix density to a more global view. The local view supposes that the *leu-500* promoter must be located directly within the domain of negative supercoiling to be activated. In the global view, unbalanced relaxation of transcriptional-induced supercoiling from the *tetA* gene results in a net reduction in the linking difference of the plasmid. If the *tetA* gene is the primary generator of supercoiling (because of its membrane anchoring effect), then it will create local domains of negative and positive supercoiling. If only the latter can be relaxed in a *topA* cell, the overall effect will be to lower the linking number of the plasmid. If the *leu-500* promoter is responding to this global change in topology, then it will do so independent of relative orientation or separation.

We arrive at the same conclusion following a second line of argument. As we discussed in the introduction, an alternative role of membrane anchorage by coupled transcription, translation, and insertion of TetA could be to provide a topological barrier so that the domains of positive and negative supercoiling generated by transcription (in theory from any promoter) cannot diffuse around the circular plasmid and undergo self-cancellation by a simple rotation of the helix. If this were true, it would require the existence of a second barrier on the opposite side of the circular plasmid, and it has been suggested that the replication origin might function in this way (19). The combined effect of two such barriers would effectively isolate the lower half of the plasmid in topological terms. However, in pL500TR, the promoter of the reversed *tetA* gene would be located in this domain, isolated topologically from the *leu-500* promoter. Yet we have shown that the single most important factor on the plasmid for the *topA*-dependent activation of the *leu-500* promoter is the *tetA* promoter. We therefore conclude that it cannot be located in a separate domain and that the barrier model does not hold. We are left with the primary role of membrane anchorage as the provision of rotational hindrance to RNA polymerase transcribing the *tetA* gene. Since the *tetA* and *leu-500* promoters are separated by more than 1.6

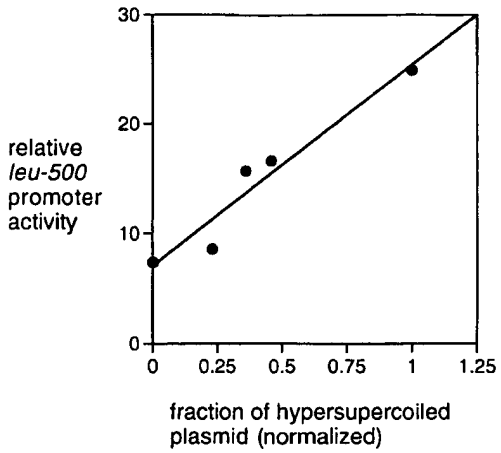

FIG. 7. **Correlation between the activity of the *leu-500* promoter and the fraction of hypersupercoiled plasmid DNA.**

kbp, this must be considered as an essentially global phenomenon in the plasmid.

The global view of the activation is consistent with measurement of the linking difference of isolated plasmids (*e.g.* Fig. 5), which is a measure of the global topology by definition. This shows that the fraction of hypersupercoiled plasmid DNA is generated whenever the *tetA* gene is present in *cis*, whatever its orientation. Indeed, we obtain a linear correlation between the level of activation of the *leu-500* promoter in *topA E. coli* with the fraction of hypersupercoiled plasmid DNA isolated from the cells (Fig. 7). *In situ* probing of the formation of cruciform structures by alternating adenine-thymine ($(AT)_n$) sequences can be used as a means of testing local negative superhelix density in cellular DNA (20), and we have shown that reporter $(AT)_n$ sequences introduced in the region corresponding to that upstream of *tetA* in pLEU500Tc detect unconstrained oversupercoiling in *topA* strains (21). However, contrary to initial expectations, we also detected elevated negative supercoiling at $(AT)_n$sequences placed downstream of the *tetA* gene,[2] *i.e.* in the region that might be expected to experience transcriptional induction of positive supercoiling. Once again this result is more consistent with a global view of the induction of negative plasmid supercoiling in *topA* cells.

The *topA*-dependent activation of the *leu-500* promoter in pL500TR does differ in some respects from that in the original pLEU500Tc containing the clockwise *tetA* gene. One is the effect of *bla* expression; we observed that *bla* deletion lowered the level of *leu-500* promoter activation in pLEU500Tc (18), whereas there is little influence of *bla* in the presence of the anticlockwise *tetA* gene of pL500TR. However, we found that the effect of *bla* deletion on the *leu-500* promoter in pLEU500Tc could be removed when a *tac* promoter was introduced into this plasmid, suggesting that subtle effects may occur in this region. Another difference is the effect of spacing. When we introduced random DNA fragments between the *leu-500* and *tetA* promoters of pLEU500Tc, this reduced the level of initiation of transcription at the former, whereas in pL500TR, the crucial $P_{tet.rev}$ is almost diametrically opposite to the *leu-500* promoter. At present we are unable to account for this difference.

There have been reports of activation of the *leu-500* promoter in *topA* cells using plasmids that do not include the *tetA* gene (22, 23). We find these observations perplexing, because in our experiments the role of the *tetA* gene is paramount. It is conceivable that other factors play a role in these constructs, but it is possible that the overall level of activation of transcriptional initiation was lower in those investigations. It is beyond question that in the plasmids based upon pLEU500Tc, the role of the *tetA* gene is essential for the observed level of activation and cannot be replaced by any other gene that we have explored. Moreover, correlation with the physical level of hypersupercoiling in our plasmids has been independently confirmed by the experiments of Mojica and Higgins (24), who measured the level of unconstrained plasmid supercoiling using an intercalation assay.

In summary, the *leu-500* promoter is activated highly efficiently in *topA* cells when it is borne on a plasmid carrying the *tetA* gene in *cis*, irrespective of orientation. The most probable explanation is that it is activated by negative supercoiling arising from the transcription of the *tetA* gene and that this process is most effective when RNA polymerase is effectively tethered due to coordinate transcription, translation, and membrane insertion. The coupling between the promoters can be fully explained by topological effects operating within the plasmid globally.

Acknowledgments—We thank Dr. Richard Bowater for discussions and the Medical Research Council and Cancer Research Campaign for financial support.

[2] R. P. Bowater, D. Chen, and D. M. J. Lilley, unpublished data.

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Circadian rhythms in prokaryotes: Luciferase as a reporter of circadian gene expression in cyanobacteria

(biological clocks/bioluminescence/*Synechococcus*)

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ABSTRACT **We have used a luciferase reporter gene and continuous automated monitoring of bioluminescence to demonstrate unequivocally that cyanobacteria exhibit circadian behaviors that are fundamentally the same as circadian rhythms of eukaryotes. We also show that these rhythms can be studied by molecular methods in *Synechococcus* sp. PCC7942, a strain for which genetic transformation is well established. A promoterless segment of the *Vibrio harveyi* luciferase structural genes (*luxAB*) was introduced downstream of the promoter for the *Synechococcus psbAI* gene, which encodes a photosystem II protein. This reporter construction was recombined into the *Synechococcus* chromosome, and bioluminescence was monitored under conditions of constant illumination following entrainment to light and dark cycles. The reporter strain, AMC149, expressed a rhythm of bioluminescence which satisfies the criteria of circadian rhythms: persistence in constant conditions, phase resetting by light/dark signals, and temperature compensation of the period. Rhythmic changes in levels of the native *psbAI* message following light/dark entrainment supported the reporter data. The behavior of this prokaryote disproves the dogma that circadian mechanisms must be based on eukaryotic cellular organization. Moreover, the cyanobacterial strain described here provides an efficient experimental system for molecular analysis of the circadian clock.**

Despite decades of study, the biochemical mechanism of circadian clocks remains a mystery. Circadian rhythms have been found in a wide spectrum of organisms (1) but, until recently, only in eukaryotes (2, 3). In the last few years circadian rhythms have been reported in the prokaryotic cyanobacteria (4–7). Unfortunately, these studies employed genetically intractable cyanobacterial strains and laborious assays to detect the rhythms. These difficulties have impeded the demonstration that the prokaryotic rhythms are equivalent to the circadian rhythms of eukaryotes.

Proof that prokaryotes have circadian pacemakers has threefold significance. (*i*) With regard to the evolutionary emergence of circadian behavior: Can the simpler organization of prokaryotes support a circadian mechanism? Is circadian behavior adaptive for prokaryotic niches as well as for eukaryotic niches? (*ii*) The previous failure of attempts to discover circadian clocks in prokaryotes has led to a "eukaryotes-only" dogma which limited the types of models that have been considered for the underlying clock mechanism (3). Now that prokaryotic cellular organization appears to be fully competent to generate circadian oscillations, a broader range of mechanisms can be seriously evaluated as candidates for the circadian pacemaker. (*iii*) The realization that prokaryotes express circadian behavior is significant from the perspective of designing an optimal strategy to discover the hitherto elusive secret of the circadian mechanism. That is, if prokaryotes display the phenomenon, then progress in elucidating its basis will probably be most rapid while using an appropriately chosen prokaryotic model; in prokaryotes, the mechanism itself may be simpler, and the average size of prokaryotic genomes, which is smaller than that of eukaryotic genomes, facilitates the goal of saturation mutagenesis for clock-related genes.

To reap the strategic benefits that prokaryotes offer, however, we must identify an organism which is amenable to molecular and genetic analysis and which exhibits circadian rhythms of a parameter that can be assayed continuously for many cycles by an automated system. We have developed such a strain by transforming a genetically tractable cyanobacterium, *Synechococcus* sp. strain PCC7942, with bacterial luciferase genes which function as a reporter of clock-controlled expression of the endogenous *psbAI* gene. The bioluminescence rhythm expressed by this reporter strain, called AMC149, is easily assayed by an automated monitoring system. The rhythm of AMC149 satisfies all three criteria of circadian rhythms (1, 2, 8, 9): persistence under constant conditions, entrainability by light/dark signals, and temperature compensation of the period.

MATERIALS AND METHODS

Strains and Growth Conditions. Wild-type *Synechococcus* sp. strain PCC7942 was grown in BG-11 medium (10) as modified by Bustos and Golden (11). Strain AMC149, which contains a modified Ω cassette (12), was cultured in the presence of spectinomycin sulfate (40 μg/ml). AMC149 was generated by transformation of wild-type *Synechococcus* with plasmid pAM977.

Construction of the Reporter Plasmid and Strain AMC149. Most restriction and modifying enzymes were purchased from Boehringer Mannheim and used as directed by the manufacturer. *Escherichia coli* strain DH5αMCR (Bethesda Research Laboratories) was the host for all plasmids. Transformants were propagated on LB (13) or Terrific Broth (14) medium in the presence of antibiotics at standard concentrations (13).

A 2.1-kb *Hin*dIII–*Sal* I fragment from pDH140 (15) containing upstream flanking sequences, the promoter (P_{psbAI}), and the amino-terminal coding region of *psbAI* (15) was ligated into *Hin*dIII/*Sal* I-cleaved pLAV1, which contains *luxAB*. Plasmid pLAV1 was constructed by L. Chlumsky as a derivative of pTB7 (16) (gift of T. Baldwin). The P_{psbAI}::*luxAB* fusion was excised from the resulting plasmid,

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Abbreviations: LL, constant illumination; LD, light/dark.

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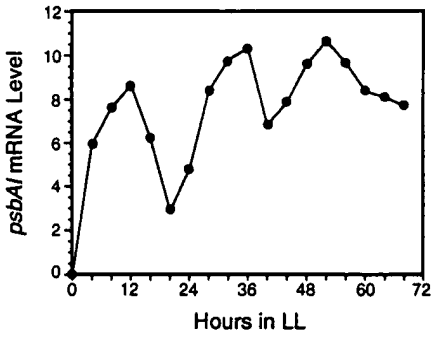

FIG. 1. Rhythmic abundance of *psbAI* message following entrainment to a LD cycle. Stationary-phase cells were entrained to a 12 hr/12 hr LD cycle as described in *Materials and Methods*. Aliquots were removed for RNA isolation at the given time points after release into LL. RNA samples (5 μg each) were subjected to electrophoresis, blotted onto a nylon membrane, and probed with a radiolabeled antisense RNA that recognizes the *psbAI* transcript. Band intensities from an autoradiogram were determined by a densitometer (Bio-Rad 620) and these values in arbitrary units were plotted versus time.

pAM976, as a 2.8-kb *Pvu* II fragment (having ≈150 bp upstream of P_{psbAI}); it was inserted into the *Synechococcus* neutral-site vector, pAM854 (11), at a *Sma* I site upstream of the Ω cassette, to produce pAM977.

Wild-type *Synechococcus* was transformed by pAM977, yielding spectinomycin-resistant colonies. These transformants carried the P_{psbAI}::*luxAB* fusion in the neutral site of the chromosome, as a result of homologous recombination, and lacked vector sequences. Southern analysis confirmed that the recombination event had occurred as predicted (data not shown).

Northern Blot Analysis of RNA from Light/Dark-Cycle-Entrained Cells. Wild-type *Synechococcus* cells were grown to stationary phase in modified BG-11 medium bubbled with 1% CO_2 in air under conditions of constant illumination (≈250 μmol·m^{-2}·s^{-1} outside of the carboy). The culture was transferred to a 30°C constant-temperature chamber, where it was stirred and provided with constant illumination for two more days (50 μmol·m^{-2}·s^{-1} outside of the carboy). The light regime was then adjusted to 12 hr of light at 50 μmol·m^{-2}·s^{-1} followed by 12 hr of darkness for five light/dark (LD) cycles. Samples were removed for RNA extraction at specific times. RNA was isolated and transferred to nylon membranes as described (17) and then probed with a gene-specific antisense RNA that recognizes the *psbAI* message (18).

Assay of the Bioluminescence Rhythm. The transformed strain AMC149 was grown in a 12 hr/12 hr LD cycle with continuous shaking (100 rpm). The light fluence rate was 46 μmol·m^{-2}·s^{-1} during illuminated periods and temperature was maintained at 30°C. When the culture reached stationary phase (≈10^9 cells per ml), 5 ml of cell suspension was transferred to a 20-ml vial. An open, sterile microcentrifuge tube containing *n*-decanal dissolved in dimethyl sulfoxide was placed upright in the vial. The vial was sealed to allow the volatile *n*-decanal to escape into the gas phase at a constant limited rate; then it was placed in front of a photomultiplier tube (Hamamatsu 931B) without shaking. The sample and photomultiplier detector were enclosed in a light-tight box. A microcomputer controlled the box to open for 25 min to illuminate the culture at 46 μmol·m^{-2}·s^{-1} and then to close for 5 min. Bioluminescence from the cells was measured during the 5-min dark period (2 min of darkness to allow chlorophyll fluorescence to decay, then two 1.5-min measurements of bioluminescence). Usually measurements were started 12 hr after the transfer of culture to the vial, to avoid a period of transient changes in physiological state. Measurements were repeated every 30 min for 1 week under continuous illumination (LL) with 5-min dark pulses as described above. Brief, repetitive dark pulses can have minor effects on circadian clocks but do not cause major interference (19). Output of the photomultiplier tube was amplified electronically and read by a microcomputer through an analog-to-digital converter. The reading was averaged over the measurement period and plotted on the ordinate in millivolts as "Bioluminescence." One unit (1 mV) roughly corresponds to a light emission of 10^5 photons per second from the vial. For the experiment shown in Fig. 4 we used a multichannel measuring assembly in which a housing that contained the photomultiplier tube moved along a guide rail to measure bioluminescence sequentially from in-line-placed vials (19). The measuring protocol was the same as for the single-channel system. Both measuring assemblies were placed in a temperature-controlled chamber to maintain constant temperature of the vials.

RESULTS AND DISCUSSION

A previous study suggested that photosynthetic activity is rhythmic in cyanobacteria (5). We reasoned that photosynthesis genes might be regulated by a circadian clock to achieve efficient photosynthesis. *Synechococcus* cells were grown to stationary phase, entrained to a LD cycle, released into constant light (LL), and sampled for RNA extraction. Northern (RNA) blot experiments indicated that the *Synechococcus psbAI* message exhibits rhythmic changes in abundance in constant conditions (Fig. 1). This message encodes D1, a major protein component of the photosystem II reaction center.

We capitalized upon the observation by fusing this gene's promoter to a reporter gene encoding bacterial luciferase. We chose this reporter because its activity (bioluminescence) can be assayed repeatedly without perturbing the cells, and its hydrophobic and volatile substrate, *n*-decanal, easily diffuses into *Synechococcus* cells at a constant rate when cultures are exposed to *n*-decanal vapor. Bioluminescence is a convenient parameter to monitor as an overt rhythm for circadian oscillators, as has been demonstrated in the naturally bioluminescent dinoflagellate *Gonyaulax* (8). We fused a promoterless segment of the wild-type *Vibrio harveyi* luciferase

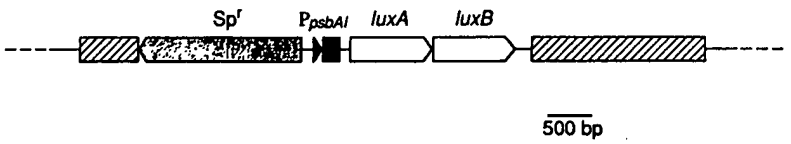

FIG. 2. Schematic representation of reporter vector pAM977, which carries a P_{psbAI}::*luxAB* transcriptional fusion. Arrows represent the coding regions of the spectinomycin/streptomycin-resistance gene (Sp^r) and the *luxAB* genes and indicate the direction of transcription for each. The *psbAI* promoter is designated as P_{psbAI} (filled triangle), and it is followed by a segment of *psbAI* amino-terminal coding region (filled rectangle) which is fused out-of-frame with *luxA*. Hatched boxes represent *Synechococcus* DNA sequences in the vector that target insertion of the cloned sequences to a locus termed a neutral site on the cyanobacterial chromosome (12). The pBR328 base of the vector is not shown, because it is not transferred to the *Synechococcus* chromosome during transformation.

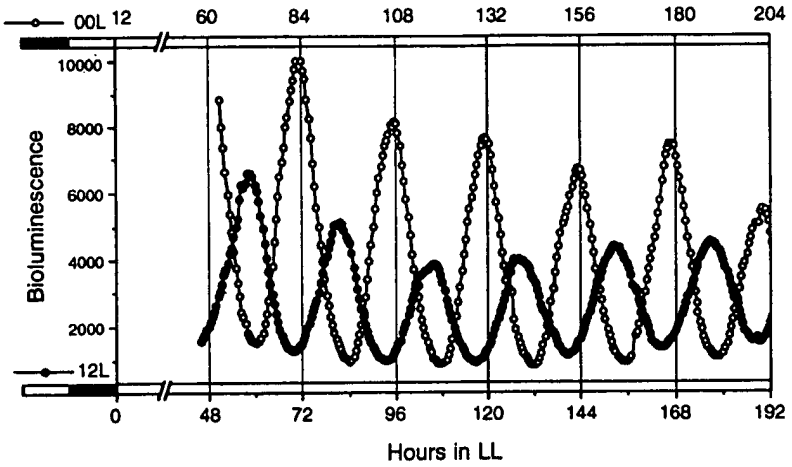

FIG. 3. Circadian rhythm of bioluminescence in continuous light conditions. The transformed strain AMC149 was cultured at 30°C under a 12 hr/12 hr LD cycle (46 μmol·m^{-2}·s^{-1}) with continuous shaking (100 rpm) and then transferred to vials for measurement of bioluminescence. The two traces are from cultures that were previously entrained to LD cycles which were 12 hr out of phase. The last LD cycles preceding LL are illustrated on the abscissa (open bar, light period; filled bar, dark period).

operon (*luxAB*) to the promoter of the *Synechococcus psbAI* gene (Fig. 2). The reporter construct was introduced into *Synechococcus* on a vector that targets cloned DNA to a nonessential region (termed a neutral site) on the chromosome (12). The resulting transformed strain was designated AMC149.

Fig. 3 shows the time course of bioluminescence in LL measured concurrently from two AMC149 cultures which had been entrained previously to LD cycles that were 12 hr out of phase. Under constant conditions, the bioluminescence from both cultures oscillated with a period of ≈24 hr, but with opposite phases. These results indicate that the bioluminescence rhythm is an expression of an endogenous clock within the cells and that the phase of the rhythm is determined by the LD cycle which precedes the continuous conditions. The peak of bioluminescence occurred 12 hr after the onset of the LL and then at 24 hr intervals. Addition of a photosystem II inhibitor, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (1 μM), or an inhibitor of bacterial translation, chloramphenicol (10 μM), abolished the rhythm. However, the eukaryotic translation inhibitor cycloheximide (35 μM) had no effect. This indicates that the rhythm originated with the cyanobacterium and was not contributed by a signal from a contaminating eukaryotic organism.

Single light or dark signals can reset the phase of circadian rhythms to a new phase, and the direction of the phase shift depends upon the phase at which the signal is administered (1, 2). This characteristic is crucial for the circadian clock to entrain its cellular activities to the solar LD cycle (9). Fig. 4 depicts the resetting of the bioluminescence rhythm in AMC149 by a single dark pulse: 4-hr dark pulses administered to cultures maintained otherwise in LL shifted the phases of the rhythms. The direction and magnitude of the resulting phase shift were a function of the phase of the rhythm at which the dark pulse was given; variation between the phases of control cultures was <1 hr.

Another criterion of circadian rhythms is that their period length is approximately constant at different ambient temperatures, a phenomenon known as temperature compensation of the period (1, 2). Fig. 5 shows that the bioluminescence rhythm of AMC149 meets this criterion: period length was 25.5 hr at 25°C, 24 hr at 30°C, and 23 hr at 36°C. The calculated Q_{10} value for frequency (1/period) of this rhythm is 1.1. This value lies within the range for the temperature dependence of circadian rhythms in eukaryotes and is far from that of most biological processes, such as growth or respiration, for which the Q_{10} values are usually between 2 and 3. Temperature compensation of the amino acid uptake rhythm has been reported for another cyanobacterium, *Synechococcus* RF-1 (7). Therefore, the rhythms of *Synechococcus* satisfy the three fundamental criteria for circadian rhythms.

Does the rhythm of bioluminescence in AMC149 prove that this promoter is under circadian control? Not necessarily. Because bacterial luciferase catalyzes the following reaction,

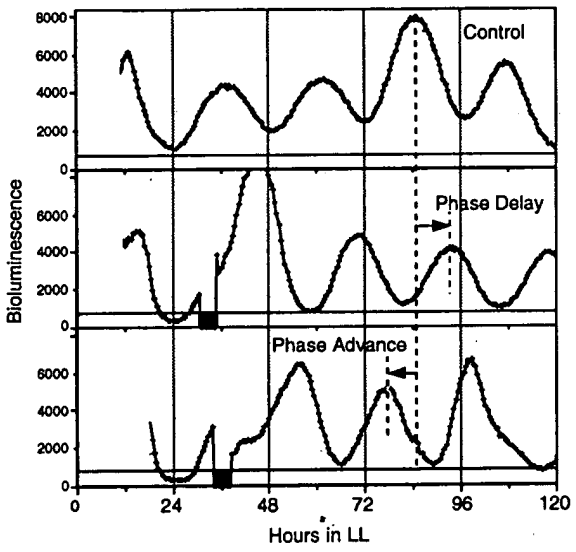

FIG. 4. Resetting of the bioluminescence rhythm by dark pulses. Three cultures were entrained to 12 hr/12 hr LD cycles. At time 0, each culture was released into LL (open bar). A single dark pulse of 4 hr (black bar) was given to the second and third cultures at the times indicated (for the middle panel, beginning 30 hr after the onset of LL; for the bottom panel, beginning 34 hr after LL onset). After the pulse, the cells were returned to LL for the duration of the experiment. The dark pulses "delayed" the phase of the rhythm by 8 hr (middle) or "advanced" it by 6 hr (bottom). Assignment of phase shifts as "advances" or "delays" is a convenient designation, although it is arbitrary in most cases (9). Experimental procedures and presentation of results are the same as in Fig. 3.

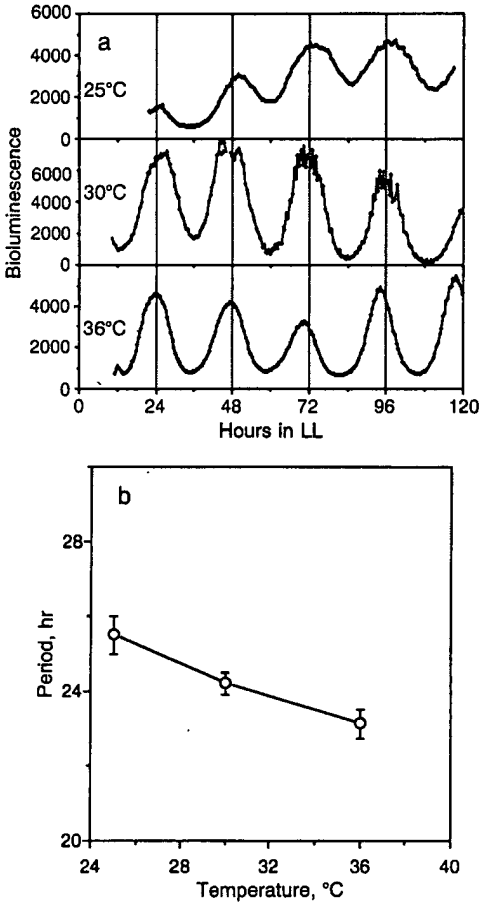

FIG. 5. Temperature compensation of the period. Experimental procedures are the same as in Fig. 3, except that the bioluminescence monitored at three temperatures is shown. (*a*) Rhythms of bioluminescence. (*b*) Periods of the rhythm vs. temperature (bars indicate standard deviation of three or four replicates).

$FMNH_2$ + *n*-decanal + $O_2 \rightarrow$ FMN + *n*-decanoic acid + H_2O + light, bioluminescence can be changed not only by changes in the intracellular level of luciferase but also by the levels of substrates. Since *n*-decanal was supplied exogenously, its effective level should be constant, but the levels of $FMNH_2$ and O_2 are unknown. To address this question, we extracted luciferase from cells cultured in LL and measured the activity *in vitro*. The activity of luciferase at the phase of maximum bioluminescence (hour 60 of LL) was 2.5 times higher than that at the minimum phase (hour 48 of LL). Note that *psbAI* mRNA levels in nontransformed cells also changed rhythmically in LL (Fig. 1). The peaks of the rhythm in mRNA level occurred at approximately the same times in LL as in the bioluminescence rhythms, that is, at hour 12, 36, and so on. Therefore, although levels of $FMNH_2$ and O_2 could be changed by photosynthesis or respiration, it is most likely that the bioluminescence rhythm exhibited by AMC149 is due to rhythmic changes in the intracellular level of luciferase which is caused by a circadian regulation of the *psbAI* promoter. Since the D1 protein plays a critical role in electron transfer in photosystem II, circadian regulation of the *psbAI* gene may be important in the genetic regulation of photosynthesis.

We report here that *Synechococcus* has a circadian clock whose fundamental characteristics are the same as those exhibited by eukaryotes. The presence of a circadian clock in cyanobacteria suggests that circadian mechanisms evolved much earlier than has been previously assumed: the first circadian system may have evolved before the branching of eubacteria and eukaryotes in the universal phylogenetic tree (20).

The molecular mechanism of circadian behavior can now be dissected in a prokaryote by using luciferase as a reporter by which to continuously monitor the rhythm. A recent report demonstrates that firefly luciferase can be used to assay circadian rhythmicity in *Arabidopsis* (21). The advantage of using bacterial luciferase in *Synechococcus* is that cells can be continuously exposed to decanal vapor without any perturbation, whereas firefly luciferase requires periodic administration with fresh luciferin substrate. Therefore, using a bacterial luciferase reporter allows us to automatically assay the bioluminescence rhythm of an undisturbed culture at a high enough frequency to gauge the waveform of a circadian output with high precision. Furthermore, because *Synechococcus* is a unicellular prokaryote with well-developed gene transfer techniques (22), its clockwork should be simpler to analyze than that of a multicellular eukaryote. Automated monitoring makes AMC149 an ideal strain for identifying and analyzing mutants affected in the function of the circadian clock.

We acknowledge the contributions of Dr. M. R. Schaefer, whose observations of *psbA* message levels first suggested circadian rhythmicity in *Synechococcus* sp. strain PCC7942. We thank Prof. J. W. Hastings (Harvard University) for his advice and the use of his bioluminescence-monitoring apparatus in early phases of this study. We thank N. Yamaguchi for constructing the monitoring device used for Fig. 4. We are grateful for helpful discussions with Dr. T.-H. Chen (Academia Sinica, Taiwan), Dr. S. Kay (University of Virginia), and Dr. S. Itoh (National Institute for Basic Biology) and to Drs. G. Shaedel and T. Baldwin for providing us with the *luxAB* genes. This research was supported by grants from the Naito Foundation (Tokyo) and the Ciba-Geigy Foundation (Japan) to T.K., the Nissan Foundation (Tokyo) and Tokai Foundation to M.I. and T.K., the National Institute of Mental Health to C.H.J. (MH43836) and to W.T. (MH46660), and the National Institutes of Health (GM37040) and American Cancer Society (JFRA-224) to S.S.G.

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